Mechanical loading and cartilage physiology

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MECHANICAL LOADING AND CARTILAGE PHYSIOLOGY

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Statement of Originality

The accompanying thesis submitted for the degree of Doctor of Philosophy is entitled “Mechanical Loading and Cartilage Physiology”. This thesis is based on the work conducted by the author in the Department of Human Health Sciences, University of Westminster during the period between October 2007 and December 2010. All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references. This work has not been submitted for another degree in this or any other University.

List of Publications

Published Conference Abstracts

1. Parker E., Getting S.J., Hucklebridge F., Vleck V.E., and Kerrigan M.J.P. The effects of REV5901 on chondrocyte viability following mechanical trauma. Bors, Newcastle, 2009.

2. Parker E, Getting SJ, Hucklebridge F, Vleck V & Kerrigan MJP. The effects of REV5901 on chondrocyte viability following mechanical trauma. Proceedings of the British Pharmacological Society, Vol 7, Issue 4, abst127P, London, UK, 2009.

3. Parker E, Noureen S, Getting SJ, Vleck VE, Hucklebridge F & Kerrigan MJP. REV5901: Chondroprotective Effects Post Mechanical Trauma. Proceeding of the Physiological Society Meeting 19, PC156, University of Manchester, UK, 2010.

4. Parker E, Domah S, Getting SJ, Vleck VE, Hucklebridge F & Kerrigan MJP. Knee Forces And Inflammation: The Effects of Foot Strike Patterns And Footwear. Proceeding of the Physiological Society Meeting 19, PC176, University of Manchester, UK, 2010.

5. Parker E, Begum N, Wunderlich C, Ravichandran N, Getting SJ, Vleck VE, Hucklebridge F & Kerrigan MJP Chondroitin And Glucosamine Supplementation Results In Increased Chondrocyte Viability Post Mechanical Trauma In Vitro. Proceeding of the Physiological Society Meeting 19, PC268, University of Manchester, UK, 2010.

6. Parker E, Qusous A, Getting SJ, Hucklebridge F & Kerrigan MJP. Cartilage And Macrophages: Evidence For A Paracrine Loop.

Proceeding of the Combined Meeting of Orthopaedic Research Societies, Poster No. 278, Kyoto, Japan, 2010.

7. Parker E, Getting SJ, Hucklebridge F & Kerrigan MJP. Diurnal Inflammatory Cytokine Changes Post-Exercise. Proceeding of the Combined Meeting of Orthopaedic Research Societies, Poster No. 319, Kyoto, Japan, 2010.

8. Parker EL, Peetroons C, Getting SJ, Hucklebridge F & Kerrigan MJP. Tamoxifen: chondroprotective effects post-mechanical trauma. Proceeding of the Physiological Society Meeting 21, PC33, University of Durham, UK, 2010.

9. Parker E, Qusous A, Getting SJ, Hucklebridge F & Kerrigan MJ. REV5901 Induces Calcium Dependant Chondroprotective Effects Post In Vitro Mechanical Loading. Proceeding of the ORS Annual Meeting Vol. 36, Long Beach, CA, 2011

Scientific Papers Submitted

1. Parker E, Qusous A, Geewan C, Kapasi A, Getting SJ, Hucklebridge F, Keshavarz T & Kerrigan MJP. Novel methods for the quantification of changes in actin organisation in chondrocytes using fluorescent imaging and linear profiling.

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Abbreviations:

$[Ca^{2+}]_0$:	extracellular calcium
$[Ca^{2+}]_i$:	intracellular calcium
2D:	Two-Dimensional
3D:	Three-Dimensional
5-LO:	5-Lipoxygenase
ADAMTS:	A Disintegrin And Metalloproteinase with Thrombospondin Motifs
AF:	Active Females
AM:	Active Males
AM:	Ante Meridiem
ATP:	Adenosine Trisphospste
AVD:	Apoptotic Volume Decrease
Bak:	Bcl-2 homologous antagonist/killer
Bax:	Bcl-2-associated X protein
Bcl-2:	B-Cell Lymphoma 2
BP:	Blood Pressure
BPS:	Basic Physiological Saline
BSA:	Bovine Serum Albumin
Ca^{2+}***:***	Calcium
$CaCl_2$***:***	Calcium Chloride
Cl^-***:***	Chloride
CLSM:	Confocal Laser Scanning Microscope
Col2:	Collagen Type II
Cyt c:	Cytochorome C
ddH_2O***:***	Double Distilled Water
DISC:	Death Inducing Signalling Complex
DMEM:	Dulbecco's Modified Eagle Medium
DMSO:	Dimethyl Sulphoxide
DNA:	Deoxyribonucleic Acid
DZ:	Deep Zone
ECM:	Extracellular Matrix
EDTA:	Ethylene-diamine-tetraacetic acid
EGTA:	Ethylene-glycol-tetraacetic acid

ELISA: Enzyme-linked immunosorbent assay

F-actin: Filamentous Actin

FADD: Fas Associated Death Domain

FCS: Foetal Calf Serum

G-actin: Globular Actin

GRF: Ground Reaction Force

H_2SO_4: Sulphuric Acid

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HR: Heart Rate

IIAD: Impact Induced Actin Decrease

IIVD: Impact Induced Volume Decrease

IL: Interleukin

IP_3: Inositol trisphosphate

K^+: Potassium

K_{ATP}: ATP-sensitive potassium channel

KCC: Potassium Chloride Co-transporter

KCl: Potassium Chloride

LTB_4: Leukotriene B_4

MDL: Maximal dominant leg

$MgCl_2$: Magnesium Chloride

MCP: Monocyte Chemoattractant Protein

MMP: Matrix Metalloproteinase

mOsm: Milliosmole/Kg H_2O

MZ: Mid Zone

NaCl: Sodium Chloride

NCX: Sodium Calcium Exchanger

NF-κβ: Nuclear Factor κβ

NH_4Cl: Ammonium Chloride

NO: Nitric Oxide

OA: Osteoarthritis

P/S: Penicillin Streptomycin Solution

PBS: Phosphate Buffer Saline

PFA: Paraformaldehyde

PG: Proteoglycan

PGE_2: Prostaglandin E_2

PI: Propidium Iodide

PKC: Protein Kinase C

PLCβ_3: Phospholipase C β_3

PM: Post Meridiem

PMN: Polymorphonuclear

PQ: Parker Qusous Formula

REV5901: Alpha-pentyl-3-(2-quinolinylmethoxy)-benzene-methanol

ROS: Reactive Oxygen Species

RVD: Regulatory Volume Decrease

RVI: Regulatory Volume Increase

SACC: Stretch Activated Calcium Channel

SF: Sedentary Females

SM: Sedentary Males

SS HR: Steady State Heart Rate

SZ: Superficial Zone

TMB: 3,3',5,5'-Tetramethylbenzidine

TNF-α: Tumor Necrosis Factor α

TNFR: Tumor Necrosis Factor Receptor

TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling

U73122: 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione

UK: United Kingdom of Great Britain and Northern Ireland

VGCC: Voltage Gated Calcium Channel

VO_2max: Maximal Oxygen Consumption

VSOAC: Volume Sensitive Organic Anion Channel

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Dedications

I dedicate this thesis to the memory of Stephen Robert Parker; my beautiful, talented, imaginative and kind little brother. You will be with me forever, like a handprint on my heart.

Abstract

Whilst mechanical impact is known to be essential for cartilage maintenance, it has been noted that altered joint loading and increased force may lead to cartilage degradation and increase the risk for the development of osteoarthritis (OA). This study investigated the cellular responses of chondrocytes to mechanical impact, and the effects of possible chondroprotective agents for OA preventative strategies in individuals exposed to high impact, repetitive loading. Single-impact mechanical trauma (force 1.14 N, pressure 6.47 KPa) was determined to induce biphasic decrease in cell volume to 647.38±60.38 μm^3 at 2 h and 516.52±38.86 μm^3 at 48 h, the initial phase of which was observed to be an active mechanotransduction mechanism, termed Impact-Induced Volume Decrease (IIVD), and the subsequent phase to be Apoptotic Volume Decrease (AVD). The newly defined IIVD was concluded to be dependent upon the PKC/PLCβ_3 pathway, and possibly mediated by intracellular Ca^{2+} store release and Volume Sensitive Organic Anion Channel (VSOAC) activity. Furthermore, mechanical impact was observed to induce a rapid decrease in F-actin from 1.19±0.13 MU to 0.87±0.02 MU, termed Impact-Induced Actin Decrease (IIAD) and associated with the biphasic rise in cell death at rates of 2.75±0.41 $\%.h^{-1}$ and 0.66±0.03 $\%.h^{-1}$. Both *in vivo* exercise and *in vitro* mechanical load induced a release IL-1β (20.67±2.58 % and 5.86±0.21 AU), MCP-1 (25.69±0.53 % and 1.45±0.01 AU) and IL-10 (8.97±2.40 % and 5.55±0.28 AU), with *in vivo* concentrations correlating with joint magnitude and strike patterns. Decreased levels of IL-1β and MCP-1 (to 9.60±2.34 % and 9.01±2.34 %, respectively) observed in the evening were further confirmed using a hyperosmotic-treated *in vitro* model of prolonged static-loaded cartilage with evidence for a IL-1β-dominated paracrine loop between articular cartilage and mononuclear phagocytes. *In vitro,* chondroprotective and anti-inflammatory actions of chondroitin sulphate, glucosamine sulphate, REV 5901 and Tamoxifen were associated with a reduction in pre-impact cell volume (average of 31.91±4.19 %) and increased pre-impact actin levels (average of 39.92±9.29 %). Anti-inflammatory agents, curcumin and dexamethasone exhibited less effective chondroprotective actions, via inhibition of IL-1β (average of 83.45±1.30 %) and thus apoptosis. To conclude, high impact exercise is recommended with a place for chondroprotective properties of chondroitin, glucosamine sulphate and/or curcumin in high-risk groups before OA onset.

Chapter 1: Introduction

The participation in sports and exercise are both generally acknowledged to benefit both the individual and society, in terms of physical & mental health, and socioeconomic status (Saxon *et al*., 1999). However, the safety of sporting/exercise activity from a musculoskeletal stand-point with respect to osteoarthritis (OA) is less well researched. It is hypothesised that continuous stress placed on the joints by long-term and prolonged exercise training may result in microtrauma and degeneration (Moskowitz, 1984), as observed in the development of overuse injury (Gosling *et al*., 2007) and, subsequently, in *increased* risk or hastened onset of OA (Saxon *et al*., 1999, Bush and Hall, 2001b, Bush *et al*., 2005).

1.1 Cartilage and Chondrocytes

Articular cartilage (mainly hyaline cartilage) is found on the articular surfaces of long bones in diarthrodial joints (Darling *et al*., 2006). It is a dense connective tissue that results in a low friction surface for smooth joint articulation as well as a framework for bone deposition during skeletal growth (Stockwell, 1978, Gray, 2000). Articular cartilage has a high tensile strength and is resistant to pressure, thus providing effective protection against joint pressures as a result of articulation (Darling *et al*., 2006, Gray, 2000, Matthews *et al*., 1977). To support this, articular cartilage is aneural, avascular and alymphatic as these structures would be damaged during joint use (Stockwell, 1979).

1.1.1 Structure of Cartilage

Articular cartilage comprises of an extracellular matrix (ECM), which is synthesised and maintained in a constant state of turnover by the single resident cell type, the chondrocyte (Byers *et al*., 1977, Darling *et al*., 2006, Hall, 1998). The ECM is adapted to the changing environmental pressures by the balance of catabolism and anabolism of the macromolecule constituents (Archer, 2003, Lin, 2006, Urban, 1994).

The principle constituents of articular cartilage ECM are collagen and proteoglycan (PG). Collagen II (col2) is the most abundant form of collagen in healthy articular tissue, comprising ≥ 90 % of the collagen present in mature cartilage (Eyre, 1987) and two thirds of the dry weight of the tissue (Eyre, 2002). Col2 exists in highly cross-linked triple helical molecules,

which form hetropolymer complexes with other cartilage specific collagens, namely collagen IX and XI (Goldring, 2000, Mayne, 1993, Poole, 1997). Zonal changes in fibrillation pattern provide the tissue with tensile strength and the ability to resist tension (Hall *et al*., 1996a, Goldring, 2000).

PGs are negatively charged molecules, which are responsible for providing articular cartilage with the ability to resist tension (Hall *et al*., 1996a, Urban, 1994) by attracting cations and osmotically obliged water. Consequently, this induces swelling of the PGs and increased tension within the collagen network where they reside, whereby the collagen mesh resists the swelling. Maintained at a steady state, this allows the tissue to resist compression (Heinegård and Oldberg, 1989, Hall *et al*., 1996a). A core protein of aggrecan and glucosaminoglycan side chains, primarily chondroitin sulphate and keratan sulphate, comprise the PGs present in articular cartilage, which subsequently interlinks with a backbone of polymeric hyaluronic acid (Hardingham, 1992, Goldring, 2000). Hyaluronic acid itself is bound to clusters of differentiation 44 (CD 44) on the cell membrane (Urban, 1994) (*Fig. 1.1*). An interesting polymorphism of the CS1 domain on the aggrecan gene results in differing concentrations of chondroitin sulphate found in cartilage (Roughley, 2001, Doege *et al*., 1997). Decreased levels of chondroitin sulphate have been noted to be associated with both increasing age and degenerative joint disease (Anderson, 1959, Matthews, 1952, Matthews, 1953)and thus implicated in increased risk of cartilage degeneration due to impaired aggrecan function (Doege et al., 1997, Roughley, 2001).

The polyanionic nature of the PGs trapped within the collagen network creates an unusual ionic environment whereby increased cations and decreased anions are present within the interstitial fluid arising from the Donnan effect (Hall *et al*., 1996a, Maroudas *et al*., 1980). The Donnan effect describes the uneven distribution of charged ions as a result of impermeable or immobilised charged molecules, in this instance PGs (Nguyen and Kurtz, 2004). The elevated cation concentration and associated decreased anion concentration results in a raised osmotic pressure, increased compared to that in synovial fluid and plasma

(Maroudas and Bannon, 1981, Urban, 1994, Urban *et al*., 1993), resulting in osmosis and tissue swelling, subsequently resisted by the collagen network. Thus, a hydrostatic pressure of 0.2 MPa is observed at rest (Maroudas and Bannon, 1981, Urban, 1994). Upon joint loading, fluid is expelled from cartilage, inducing an increased concentration of PGs and thus an increased osmotic pressure, a consequence of the associated elevation in cation concentration and decrease in anion concentration (Hall *et al*., 1996a, James and Uhl, 2001). The osmotic pressure is increased until equilibrium with the applied load is reached, thus providing the cartilage with its compressive resistance. After removal of the load, the increased osmotic pressure induces the PGs to function hydrophilically, imbibing fluid back into the tissue in an action similar to that of a sponge soaking up water, thus preparing the cartilage for the next load (James and Uhl, 2001, Mow and Rosenwasser, 1988).

In addition to the described structural proteins, cartilage comprises several other proteins involved in the regulation of the ECM, including fibronectin and anchorin (Goldring and Marcu, 2009), whereby fibronectin, a dimeric glycoprotein, regulates cellular adhesion and differentiation (Ruoslahti, 1988), and anchorin, a member of the annexin family, is a collagen binding protein (Hofmann *et al*., 1992).

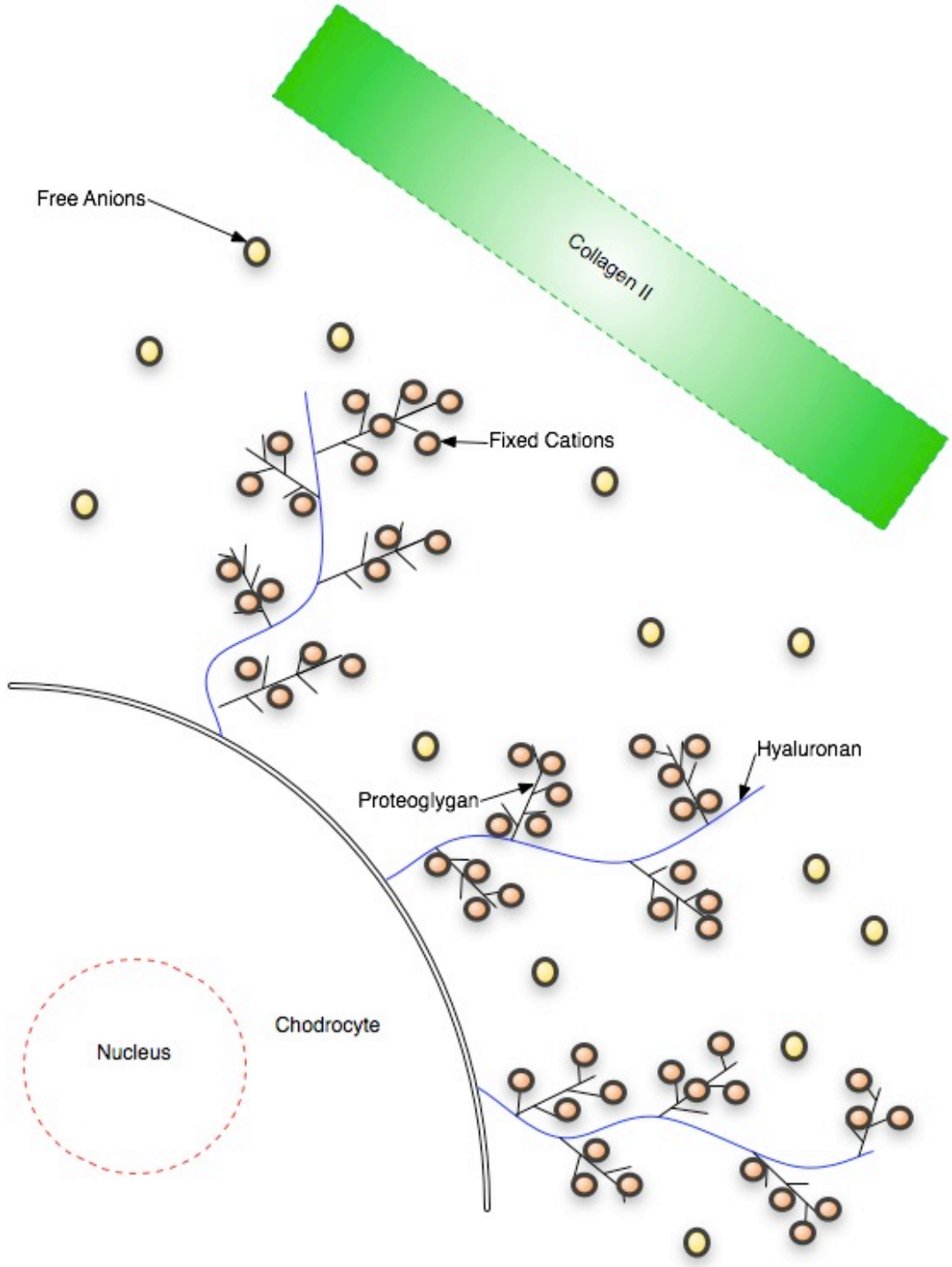


Figure 1.1: Schematic diagram of the structure of the extracellular matrix (ECM)

The main components of the ECM are collagen II (col2) fibres and proteoglycans (PGs). PGs branch from a spine of hyaluronan that extends from the chondrocyte surface. Negatively charged glucosaminglycan side branches of the PGs attract cations, resulting in osmotic pressure. Col2 fibres form a mesh frame work, interlinking with collagen IX fibres, that traps the PGs. Adapted from (Urban, 1994).

1.1.2 Matrix Regulation and Metabolism

The matrix of articular cartilage displays slow turnover and remodelling to maintain a healthy tissue (Aigner *et al*., 2006). Mechanical stress imposed on the joints induces damage to the ECM, which is compensated for by the turnover of ECM components, predominantly damaged PGs (Aigner *et al*., 2006). Within healthy cartilage, an equal balance between the anabolism and catabolism of ECM is evident, however disruption of this careful balance by tipping the impetus to catabolism results in ECM degeneration (Aigner *et al*., 2006).

Catabolism of the ECM is driven by a variety of proteinases, including matrix metalloproteinases (MMPs) and ADAMTS (A Disintegrin And Metalloproteinase with Thrombospondin Motifs). MMPs cleave ECM components, in addition to other proteinases, cell surface receptors and cell-cell adhesion molecules (Sternlicht and Werb, 2001), and are traditionally grouped according to the substrate they cleave (Nagase and Woessner, 1999). MMP-3 and MMP-10 (stromelysin-1 and -2 respectively) have a broad substrate specificity, but are noted to cleave PGs and fibronectin, similarly to ADAMTS, also termed aggrecanases, which additionally cleave PGs (Nagase and Woessner, 1999, Caterson *et al*., 2000). MMP-1, MMP-8 and MMP-13 cleave collagens, thus often referred to as collagenase-1, -2 and -3 respectively, though the specificity of collagen type differs, with MMP-13 cleaving col2 and MMP-1 and -8 cleaving collagen III and I respectively (Knauper *et al*., 1996, Cawston and Young, 2010). MMP-2 and -9, referred to as gelatinases, cleave denatured collagenases in addition to collagen IV, collagen V and elastin (Aimes and Quigley, 1995). Unsurprisingly, given the ECM degradation present in arthritic diseases, MMPs and ADAMTS are noted to be present in increased concentrations in the synovial of both rheumatoid and ostoarthritic patients (Cawston and Young, 2010, Hembry *et al*., 1995, Tetlow *et al*., 2001, Yoshihara *et al*., 2000). MMPs and ADAMTS are regulated somewhat by the endogenous production of tissue inhibitors of metalloproteinases (TIMPs), whereby TIMPs bind 1:1 with MMPs to control ECM breakdown, and inhibit ADAMTS activity (Brew *et al*., 2000, Cawston, 1996, Kashiwagi *et al*., 2001).

1.1.3 The Chondrocyte

The single resident cell type within articular cartilage is the chondrocyte. This terminally differentiated cell (Goldring, 2000) exists at a density of 10,000 chondrocytes/μm^3 in the human femoral head (Stockwell, 1971) and comprises 1-5 % of cartilage tissue volume (Stockwell, 1979). Chondrocytes sense and respond to loading to produce ECM of the required durability via a yet to be fully-elucidated combination of signalling (Hall et al., 1996a, Ramage et al., 2009) resulting in the synthesis and breakdown of the ECM (Stockwell, 1978). The avascular nature of cartilage ensures chondrocytes operate at low oxygen levels and rely on diffusion from the articular surface for nutrients (Rajpurohit, 1996).

Chondrocytes arrangement within articular cartilage is not homogenous, when observed in cross-section (*Fig. 1.2*), articular cartilage is revealed to be comprised of a zonal organisation featuring 4 distinct zones; superficial zone (SZ), middle zone (MZ), deep zone (DZ) and calcified zone (Stockwell, 1991). The SZ, which comprises 10-20 % of the full depth (Kim *et al*., 1994), has characteristically ellipsoid chondrocytes, arranged parallel to the synovial surface of an approximate volume of 454±18 μm^3 (Bush and Hall, 2001b) which are densely packed in surrounding collagen fibres (Stockwell, 1991). In contrast, MZ chondrocytes are observed to be spherical in shape and of a slightly larger volume of 553±15 μm^3 (Bush and Hall, 2001b). MZ chondrocytes are often arranged in pairs and are embedded in a random distribution in a “basket-weave” of collagen fibres (*Table 1.1*). In the DZ, ellipsoid chondrocytes of 805±79 μm^3 (Bush and Hall, 2001b) are arranged in columns distributed between radially orientated collagen fibres which extend from the calcified zone (Darling *et al*., 2004, Maroudas, 1979).

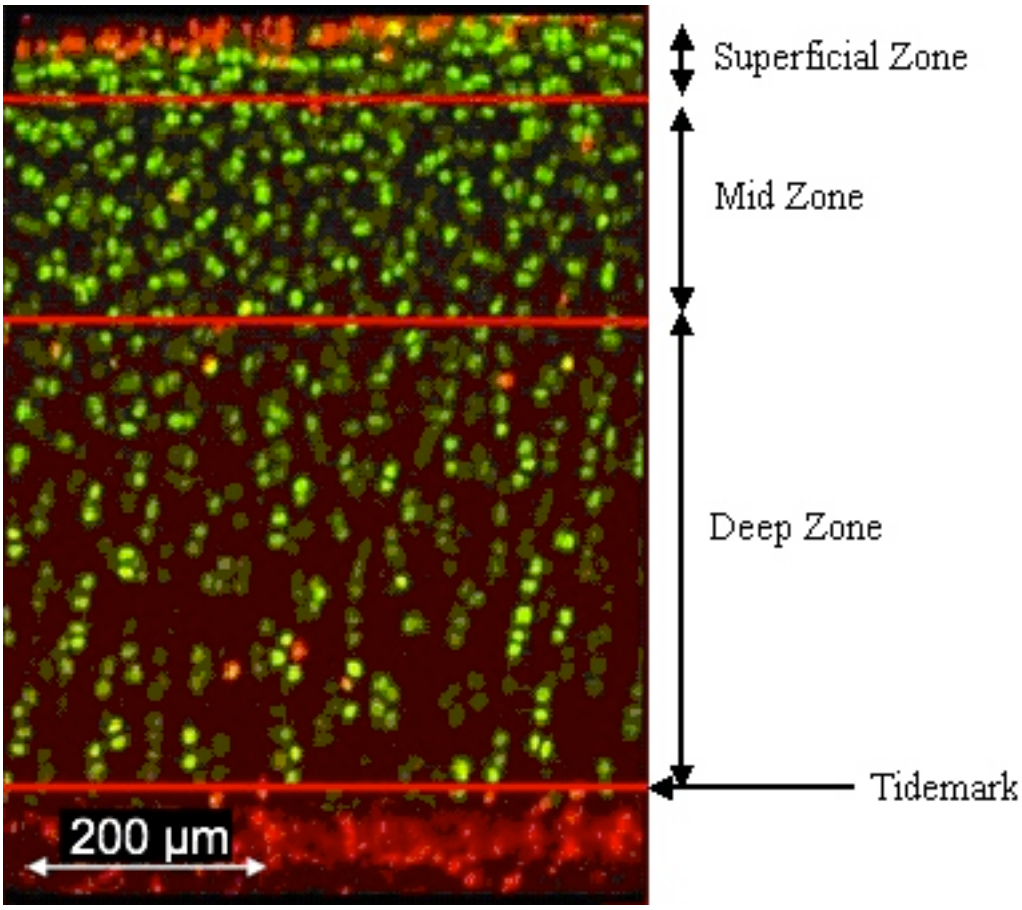


Figure 1.2: Representative zonal image of bovine articular cartilage

Full-depth cartilage explants were dissected aseptically from bovine metacarpal and metatarsal pharyngeal joints and loaded with 5 μM calcein-AM (green) and 1 μM propidium iodide (red). Explants were visualised using confocal laser scanning microscopy. Superficial Zone chondrocytes are aligned parallel to the articular surface, Mid Zone chondrocytes are found in pairs and Deep Zone chondrocytes in columnar stacks, perpendicular to the articular surface. Image acquired by Dr Mark J. P. Kerrigan-Holt, adapted with permission.

Zone	Chondrocyte Morphology	Volume (μm^3)	Collagen Orientation
SZ	Single, ellipsoid	454±18	Tight fibrils, aligned with shear stress
MZ	Single or paired, spherical	553±15	Random, radial 'basketweave'
DZ	Columns, ellipsoid	805±79	Perpendicular to articular surface

Table 1.1: Summary of the characteristics of zonal articular cartilage

Chondrocytes possess differing properties dependent on the Zone. Superficial Zone (SZ) chondrocytes are ellipsoid in morphology and embedded in tangentially arranged collagen fibres, whereas Mid Zone (MZ) chondrocytes have a more spherical morphology, a larger volume and found in pairs within a 'basketweave' structure of collagen fibres. Deep Zone (DZ) chondrocytes possess a larger still volume and are arranged in columns within collagen fibres which extend perpendicularly to the articular surface (Bush and Hall, 2001b) (Hall, 1998, Maroudas et al., 1980, Urban, 1994).

1.2 Osteoarthritis.

Osteoarthritis (OA), is the most common form of arthritis (Felson and Zhang, 1998, Silman, 2008), affecting 10-20% of the UK population over the age of 65 with ~8 million sufferers in the UK (ARC, 2008). It is associated with defective articular cartilage, changes in the regulation of matrix synthesis, increased hydration, changes in chondrocyte morphology and changes to the underlying bone and joint margins (Buckwalter, 1996, Panush and Lane, 1994). These changes result in a loss of protection at the synovial joint interface during the process of joint articulation and movement. The symptoms of OA include intense joint pain, decreased mobility and a decreased 'Quality of Life' (Dunlop *et al*., 2005, Hennessy *et al*., 1994). OA accounts for more dependency in walking, stair climbing and other lower extremity tasks than any other disease and more than three times that of rheumatoid arthritis (Guccione *et al*., 1994). Economically, OA's costs are a cause for increasing concern such that in the UK alone, the cost is an estimated £5.7 billion annually (mostly due to work loss and disability claims) (ARC, 2008). Therefore research into the events which initiate and progress OA are of major interest to public health (Fernandes *et al*., 2002).

OA is acknowledged to involve multiple etiolgies, including biomechanical, biochemical and genetic factors contributing to the development of OA by disrupting chondrocyte-ECM associations and metabolic processes (Goldring, 2000, Poole, 1997, Radin *et al*., 1991). Whilst biomechanical stresses are observed to be significant in OA development, with continual mechanical wear a prevalent theory behind OA, ageing is traditionally considered as the major risk factor (*Fig. 1.3*). Aged cartilage is characterised by a decrease in PGs, both in size and in concentration, and an increase in collagen cross-linking, which induce a decreased water content and increased stiffness respectively (Verzijl *et al*., 2003). Additionally, a decrease in the anabolic activity of chondrocytes in aged cartilage is observed (Loeser, 2004). It is thought that loss of viable chondrocytes due to apoptosis and programmed cell death renders aging cartilage more susceptible to degeneration initiated by normal loading

(Horton *et al*., 2006, Loeser, 2008). Abnormal loading leading to mechanical trauma has been observed to increase risk of OA development (Buckwalter, 2004, Lotz, 2010) with the risk further increasing with age upon mechanical trauma and time post trauma (Gelber *et al*., 2000, Lotz, 2010, Roos *et al*., 1995). Cartilage damage resultant from trauma is irreversible, and thus is likely to be a major determinant in later OA development (Lotz, 2010). Mechanical trauma is observed to initiate localised necrosis and structural tissue damage (Otsuki *et al*., 2008) and further cell death subsequently spreads throughout the tissue (Quinn *et al*., 1998, D'Lima *et al*., 2001a). Additionally, increased IL-1 is observed post mechanical trauma. IL-1 is noted to have catabolic effects on the ECM, whereby it induces an increase in proteinases, including MMP-1, -3, -13 and ADAMTS-4 (Bau *et al*., 2002, Mengshol *et al*., 2000), and stimulates the release of additional cytokines with which IL-1 acts synergistically to promote ECM catabolism (Bender *et al*., 1990, Geng *et al*., 1996). IL-1 is additionally associated with a decrease in aggrecan and col2 expression (Goldring *et al*., 1988, Lefebvre *et al*., 1990), thus IL-1 promotes ECM catabolism whilst reducing anabolism, driving ECM degradation. Cell death, ECM degradation and increased IL-1 are all noted features of OA tissue (Kuhn *et al*., 2004, Lotz, 2010).

In OA tissue, there is a transient proliferative response in chondrocytes and increased synthesis of ECM initially, as an attempt at tissue repair (Goldring, 2000). Further to this, there is observed a local loss of PGs and cleavage of col2 at the surface of the affected tissue which leads to increased fluid and thus a decreased tensile strength (Goldring, 2000, Horton *et al*., 2006). The damaged ECM results in the production of matrix protein fragments (including, but not limited to, collagen and fibronectin) which stimulate the chondrocyte to produce inflammatory mediators and proteolytic enzymes including matrix metalloproteinases (MMPs) (*Table 1.2*) (Homandberg, 1999, Loeser, 2008).

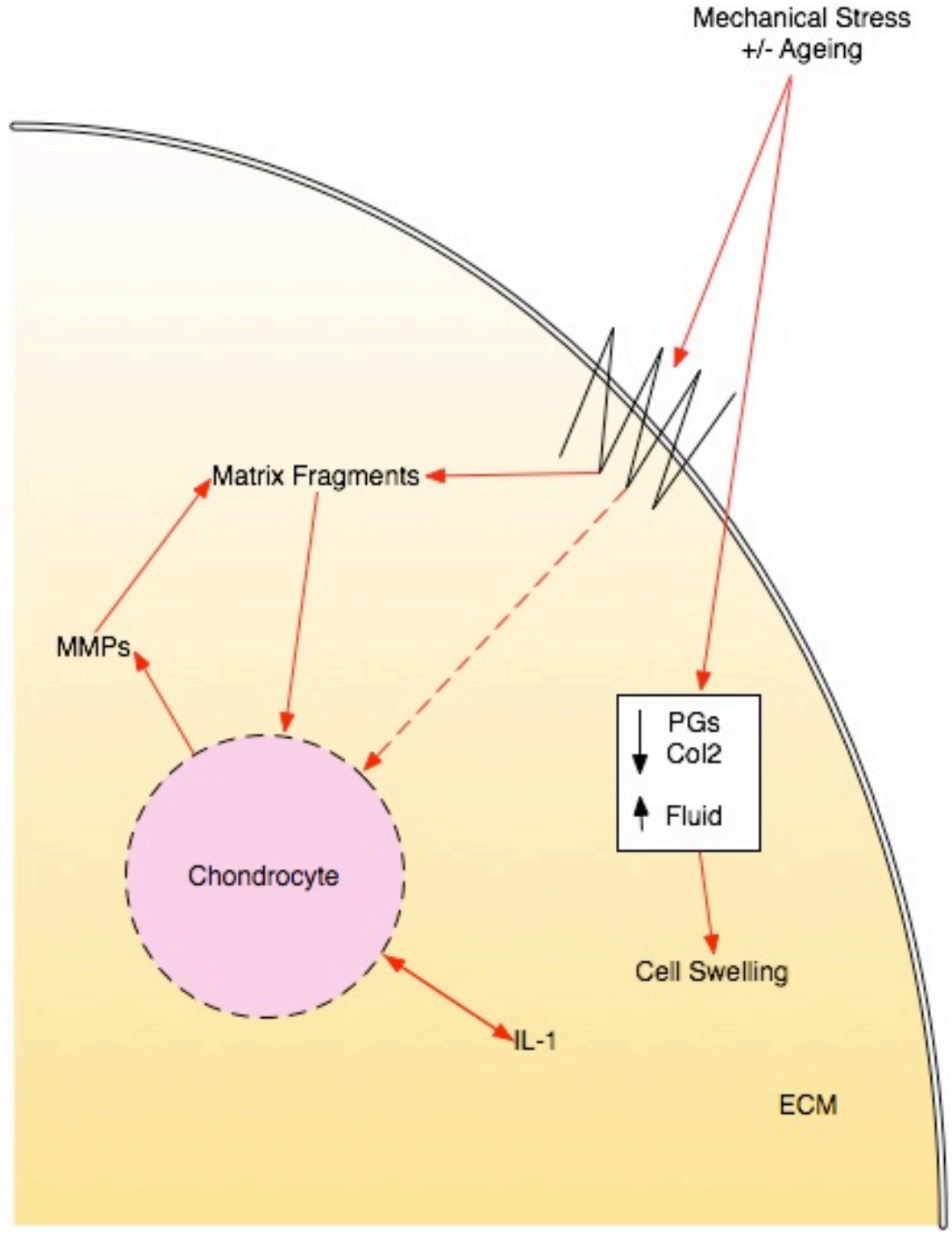


Figure 1.3: Model of the role of mechanical stress in the pathogenesis of OA

Tissue damage resultant from mechanical stress induces decreased proteoglycans (PGs), collagen II (col2) and matrix fragments. Increased fluid into the tissue due to the loss of PGs induces cell swelling. Additionally, matrix metalloproteinases (MMPs) production is increased as a result of the mechanical stress and the matrix fragments, resulting in further extracellular matrix (ECM) degradation. IL-1 is released by the chondrocytes in response to the matrix degradation and mechanical stress, further stimulating matrix degradation and additional IL-1 production. Adapted from (Goldring and Marcu, 2009).

Regulation	Function
↑(early OA) ↓ (late OA)	Collagen II and aggrecan synthesis
↑	Cell proliferation
↑	Catabolic cytokine production
↑	Proteinases (MMPs, aggrecanase, cathepsins)
↑	IL-1 receptor type I
↑	Apoptosis
↑	Collagen IIA, III, VI and X expression
↓	IL-1 receptor antagonist

Table 1.2: Characteristic functions of osteoarthritic articular chondrocytes

The initial stages of osteoarthritis (early OA) are characterised by an increase of collagen II and aggrecan synthesis as an early attempt at tissue repair. Subsequent to this, (late OA), both collagen II and aggrecan synthesis are reduced. Additionally, an increase is observed in cell proliferation, cytokine production, proteinases, cell death by apoptosis, and the expression of other collagens, including IIA, III, VI and X, and IL-1 receptors. Conversely, IL-1 receptor antagonist production is decreased (Goldring, 2000, Goldring and Marcu, 2009, Homandberg, 1999, Horton et al., 2006, Loeser, 2008).

1.3 Cytoskeletal Organisation

The chondrocyte cytoskeleton is a 3D, dynamic intracellular network comprising of vimentin, tubulin and actin. Vimentin exists within the chondrocyte as a highly organised fibrous protein filaments, observed to be interspersed within the cytoplasm, connecting the periphery of the cell to the nucleus (Fioravanti *et al*., 2003, Trickey *et al*., 2004). Whilst the exact function of vimentin is yet to be uncovered, they are thought to play a role in mechanical signal transduction, and maintaining cell mechanical integrity (Lazarides, 1980, Traub, 1995, Trickey *et al*., 2004, Wang *et al*., 1993). Vimentin filaments are increased within load bearing regions (Eggli *et al*., 1988) and whilst static load is observed to increase filament organisation, dynamic, cyclic load decreases vimentin mRNA expression (Durrant *et al*., 1999, Lahiji *et al*., 2004). Additionally, vimentin is noted to be decreased within OA chondrocytes (Lambrecht *et al*., 2008). Tubulin exists as dimeric proteins which self-assemble to form polymeric microtubules (Valiron *et al*., 2001). Within the chondrocyte, they have a loose, 'basket-mesh' uniform distribution (Fioravanti *et al*., 2003, Jortikka *et al*., 2000, Langelier *et al*., 2000, Trickey *et al*., 2004), and although are not thought to be essential to cell mechanical integrity (Trickey *et al*., 2004), they are noted to have crucial involvement within PG and collagen synthesis, whereby disruption of microtubules with colchicine inhibited PG and collagen synthesis and secretion (Jansen and Bornstein, 1974, Lohmander *et al*., 1976). Loading has no noted effects on tubulin distribution or concentration, however tubulin microtubules are observed to be absent in human OA chondrocytes (Fioravanti *et al*., 2003).

The actin cytoskeleton provides mechanical support and is involved in a vast number of cellular functions. Actin, the most abundant protein in eukaryotic cells (Disanza *et al*., 2005), is a 43 kDa globular protein monomer (termed G-actin) which forms highly organised polymer filaments (termed F-actin; *Fig. 1.4*). Polymerisation of actin has been observed to be both ATP and Mg^{2+}/Ca^{2+} dependent (Cooper and Schafer, 2000, Ono, 2007) and regulated by a collection of proteins, including nucleation promoting proteins Arp2/3 and Profilin (Goley and Welch, 2006),

depolymerising proteins ADF and Cofilin (Ono, 2007), G-actin sequestering protein Thymosin β_4 (Yarmola and Bubb, 2004) and capping protein Gelsolin (Yarmola and Bubb, 2004).

Actin is observed to have a role in a variety of cellular functions, including cell migration (Heath and Holifield, 1991), adhesion (Turner and Burridge, 1991), endocytosis (Riezman *et al*., 1996, Sontag *et al*., 1988), secretion and changes in cell shape (Sims *et al*., 1992). Within chondrocytes, the actin cytoskeleton has been noted to provide the cell with mechanical integrity required to withstand mechanical loading (Guilak, 1995). When treated with Cytochalasin D, a fungal metabolite known to inhibit actin polymerisation (Schliwa, 1982), chondrocyte stiffness was observed to be decreased by 90 %, and, conversely, viscosity to be increased by 80 % (Trickey *et al*., 2004). Additionally, Cytochalasin D treatment decreased ECM synthesis and chondrocyte anchorage to extracellular PGs (Nofal and Knudson, 2002).

F-actin is observed to be distributed cortically in *in situ* chondrocytes, predominantly localised to the periphery of the cell (*Fig. 1.5*; (Durrant *et al*., 1999, Langelier *et al*., 2000). The organisation of the F-actin filaments is known to be influenced by the osmotic environment, mechanical loading and inflammation, all of which are linked to OA. Subsequently, chondrocytes from OA tissue are noted to display an adjusted distribution of F-actin, with filaments less defined and locally diffused in the cytoplasm (*Table 1.3*). Similar to OA, in which cell swelling is observed, hypo-osmotic conditions have been observed to result in a decrease in F-actin, conversely, hyperosmotic conditions induce an increase (Guilak *et al*., 2002). Mechanical pressure has been observed to result in F-actin remodelling (Knight *et al*., 2006) in a load dependent fashion, with hydrostatic pressure of 15 MPa inducing a decrease in F-actin filaments, and 30 MPa inducing a near total disappearance of F-actin (Parkkinen *et al*., 1995). The distribution of F-actin is additionally observed to be affected by mechanical load, with a more interspersed organisation noted (Knight *et al*., 2006). Furthermore, mechanical loading induced a 20 fold transient increase in Thymosin β_4, which binds to G-actin, preventing polymerisation,

in addition to an increase in Cofilin (Blain, 2009). Finally, IL-1β has been shown to disrupt F-actin in astrocytes (Liu *et al*., 1994) and regulate cytoskeletal genes (Joos *et al*., 2008), thus it is suggested inflammatory environments, such as OA, compromise the cytoskeleton.

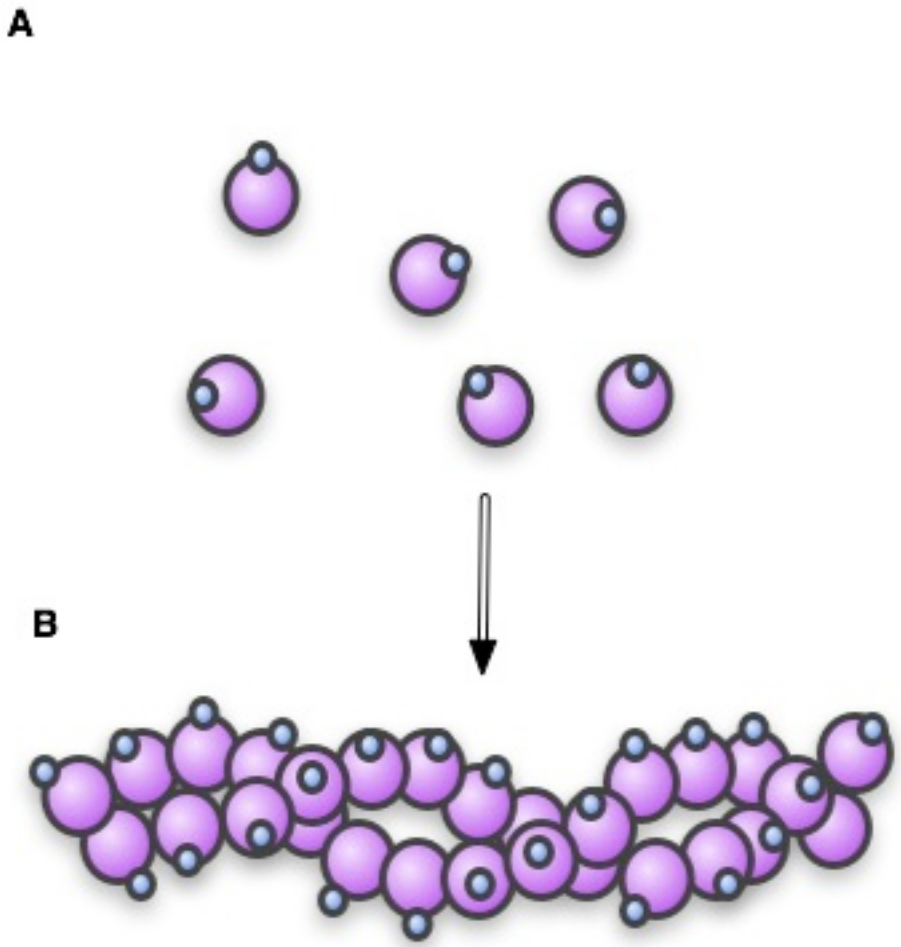


Figure 1.4: Schematic representation of the structure of cytoskeletal actin

Actin exists within the chondrocyte as globular monomeric proteins, termed G-actin (A). G-actin polymerises in an ATP and Mg^{2+}/Ca^{2+} dependent manner to form highly organised filaments termed F-actin (B; (Blain, 2009, Cooper and Schafer, 2000, Disanza et al., 2005, Hall et al., 2000, Ono, 2007). Adapted from (Blain, 2009).

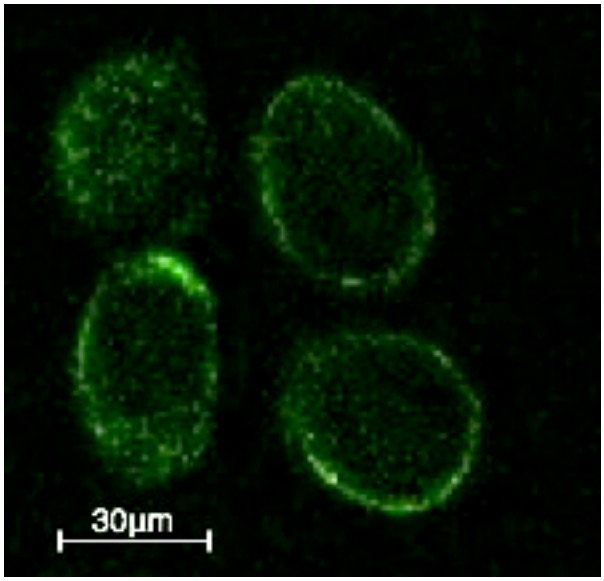


Figure 1.5: Actin organisation in *in situ* articular chondrocytes

Full-depth cartilage explants were dissected aseptically from bovine metacarpal and metatarsal pharyngeal joints. Explants were fixed using 4 % paraformaldehyde, permeabilsed using Triton x100 and subsequently labelled with 5 µl/ml Alexa-488 Phalloidin. Explants were visualised using confocal laser scanning microscopy. Actin was observed to be distributed cortically and predominantly localised to the periphery of the cell (Durrant et al., 1999, Langelier et al., 2000).

Chondrocyte	Organisation of F-actin	Reference
Healthy *in situ*	Cortical distribution, predominantly localised to the periphery.	(Blain *et al*., 2006, Langelier *et al*., 2000, Trickey *et al*., 2004, Fioravanti *et al*., 2003)
Mechanical loading	Load dependent, reversible loss of actin fibres. More interspersed arrangement.	(Blain *et al*., 2006, Knight *et al*., 2006, Langelier *et al*., 2000, Parkkinen *et al*., 1995, Trickey *et al*., 2004)
Osmotic stress	Hyperosmotic induces increased actin fibres, hypo-osmotic induces decreased actin fibres.	(Chao *et al*., 2006, Erickson, 2003)
OA	Less defined and localised diffusely in the cytoplasm.	(Fioravanti *et al*., 2003)

Table 1.3: The organisation of actin in healthy, osteoarthritic and stressed articular chondrocytes

In healthy chondrocytes, actin is distributed cortically and peripherally localised. Upon mechanical loading, a decrease in actin fibres is induced in a load dependent manner. Similarly, increased osmotic pressure reduces actin fibres, whereas in contrast, decreased osmotic pressure decreases actin. In osteoarthritic tissue (OA) actin is less defined and diffused through the cytoplasm.

1.4 Mechanical Loading

Mechanical load is essential for cartilage regulation, providing the necessary signalling for the production of an ECM of sufficient durability to withstand the stress level (Helminen, 1987). However, it has been noted that abnormal loading can lead to cartilage degradation, increasing the risk for the development of OA (Griffin and Guilak, 2005). Previous work has concluded that factors such as obesity and increased body mass have a link to an increased incidence of OA (Felson *et al*., 1988). This is thought to be a result of the increased force to the joint leading to a loss of chondrocyte function and thinning of the articular cartilage (Oliveria *et al*., 1999).

1.4.1 Mechanical Load and Cartilage Viability

Abnormal joint loading can lead to an imbalance in cartilage metabolism and thus cartilage deterioration. Excessive loading activates IL-1β pathways, promoting synthesis of catabolic proteases as well as inhibiting ECM synthesis. High impact mechanical loading results in tissue swelling, indicative of damage to the collagen network, and chondrocyte death by apoptosis and necrosis (Tew *et al*., 2000). This decreased chondrocyte viability, another feature of OA, places an increased metabolic burden on the remaining cells.

Chondrocyte cytoskeletal elements are components of mechanical signalling, whereby actin converts the mechanical stimuli to a biochemical response and vimentin is increased in areas of greatest weight bearing (Blain, 2009). Abnormal loading causes an increase in actin disassembly proteins, such as thymosin β_4, resulting in remodelling of the actin skeleton from a uniform cortical distribution to a more intermittent organisation as well as a loss of actin stress fibres (Blain, 2009). In addition mechanical load injury causes the collapse of the vimentin network around the nuclei of chondrocytes. Combined these factors result in a loss of chondrocyte function.

1.4.2 Static Loading and Cartilage Integrity

It is known that static loading compresses articular cartilage, leading to the expulsion of water from the ECM and thus raising the extracellular osmolarity of the tissue and consequently decreasing chondrocyte cell volume. Previous studies have linked matrix metabolism to cell volume and extracellular osmolarity. Both decreased osmolarity (resulting in cell swelling) and increased osmolarity (resulting in cell shrinkage), a result of prolonged static loading, significantly reduce matrix synthesis (Schneiderman *et al*., 1986, Urban *et al*., 1993). Whilst swollen cartilage (as characteristic of the early stages of OA) results in increased cartilage deformation when subjected to abnormal loading (Bush and Hall, 2001a), it is currently unknown what effect cartilage with a higher extracellular osmolarity (resulting from prolonged static loading that occurs over the course of a day) will have on cartilage integrity when subjected to similar abnormal loading.

1.5 Mechanotransduction

Chondrocytes, as previously described, are constantly subjected to varying mechanical stimuli to which they possess the vital ability to 'sense' and convert to chemical and physical responses; a process termed mechanotransduction. Thus, chondrocytes can 'respond' to the constant changes in their extracellular environment to adapt and maintain optimal intracellular environment and adapt the ECM to withstand the external pressures. Joint articulation is commonly the cause of such changes to the extracellular environment, inducing stimuli that result in a mechanotransduction response including fluid flow (Yellowley *et al*., 1997), osmotic changes (Hall, 1998), membrane deformation (Roberts *et al*., 2001) and changes in pressure (Roberts *et al*., 2001). *In Vitro* compression of cartilage has additionally been observed to induce these mechanotransduction stimuli (Hall *et al*., 1999, Hall *et al*., 1996a, Maroudas *et al*., 1980).

Pressure is applied to articular cartilage almost constantly, with pressure when walking oscillating between 0.2 and 4 - 5 MPa in the hip joint (Hodge et al., 1986). Pressure has been observed to induce deformation of chondrocytes (Broom and Myers, 1980), loss of interstitial fluid and concentration of PGs (Maroudas and Bannon, 1981, Urban, 1994), and thus increase tissue osmolarity. However, when the pressure is removed (for example during period of unloading – e.g. supine sleep), these changes can be reversed to resting equilibrium in healthy tissue (Maroudas and Bannon, 1981, Urban, 1994). Importantly, pressure induces an increase in aggrecan, col2 and matrix metalloproteinase 3 (MMP-3), thus influencing ECM metabolism, likely to generate a matrix of sufficient durability to withstand the pressure being applied (Millward-Sadler *et al*., 2000).

1.5.1 The Role of Calcium Signalling in Mechanotransduction

Calcium signalling is thought to play a role in mechanotransduction, with changes in $[Ca^{2+}]_i$ linked to fluid flow (Yellowley *et al*., 1999, Yellowley *et al*., 1997), osmotic changes (Hopewell and Urban, 2003, Kerrigan *et al*., 2006, Urban *et al*., 1993, Yellowley *et al*., 1999, Yellowley *et al*., 1997), membrane deformation (Guilak *et al*., 1999b) and pressure (Guilak *et al*., 1999a). Fluid flow has been observed to induce both an influx of Ca^{2+} into the cell via stretch-activated cation channel (SACC) activation, and release of Ca^{2+} from intracellular stores (Yellowley *et al*., 1999, Yellowley *et al*., 1997). Conversely, membrane deformation, when induced by *in vivo* fluid flow and *in vitro* mechanical load, gave rise to an increase in $[Ca^{2+}]_i$ determined to be intracellular store independent, but which was inhibited by both SACC and Sodium Calcium Exchanger (NCX) pharmacological inhibitors (Guilak *et al*., 1999b). Osmotic changes induced a similar increase in $[Ca^{2+}]_i$ (Hopewell and Urban, 2003, Urban *et al*., 1993), observed to be SACC mediated when induced by hypo-osmotic challenge (Kerrigan and Hall, 2007), as confirmed by inhibition in response to gadolinium (Gd^{3+}; a SACC inhibitor), and NCX mediated when induced by hyperosmotic challenge (Sánchez and Wilkins, 2004). Additionally, hypo-osmotic challenge induced $[Ca^{2+}]_i$ was observed to involve release from the intracellular stores (Sánchez *et al*., 2003) (*Table 1.3*).

Stimuli	$[Ca^{2+}]_i$ changes	Mediated by:
Fluid Flow	↑	Intracellular store release and SACC
Membrane Deformation	↑	SACC and NCX
Hypo-osmotic Challenge	↑	Intracellular store release and SACC
Hyperosmotic Challenge	↑	NCX

Table 1.4: Changes in $[Ca^{2+}]_i$ in mechanotransduction

Mechanotransduction stimuli induce an increase in $[Ca^{2+}]_i$ mediated by intracellular store release, stretch-activated calcium channels (SACC) or sodium calcium exchanger (NCX)(Guilak et al., 1999b, Hopewell and Urban, 2003, Kerrigan and Hall, 2007)

1.5.2 Chondrocyte Volume Regulation

Joint-loading induces chondrocyte mechanotransduction responses and cell volume changes, as detailed previously. These responses, consequently, activate volume regulatory mechanisms, including Regulatory Volume Decrease (RVD) and Regulatory Volume Increase (RVI; (Kerrigan *et al*., 2006). RVI and RVD are mechanisms by which some cell types, including chondrocytes, maintain a dynamic volume equilibrium and ionic environment necessary for optimal physiological and metabolic processes (O'Neill, 1999). Restoration of cell volume following cell swelling or shrinkage is the resultant effect of these mechanisms within healthy tissue (Hoffmann *et al*., 2007). Whilst RVI has been observed in 2D cultured chondrocytes, limited RVI response to hyperosmotic challenge has been observed in either freshly isolated or *in situ* chondrocytes (Kerrigan *et al*., 2006), indicating mechanotransduction mechanisms adapt upon culture as a result of changing phenotype. Interestingly, *in situ* chondrocytes have been observed to have a decreased cell volume after mechanical loading, compared to unloaded tissue (Buschmann *et al*., 1996, Wilkins *et al*., 2000). The lack of RVI response *in situ* is not a chondrocyte specific phenomenon, it being attributed to adverse Cl^- gradients in other cell types (Hoffmann *et al*., 1983, Wilkins *et al*., 2000). Thus it can be postulated that low Cl^- concentration with the ECM are responsible for the absence of RVI *in situ* (Wilkins *et al*., 2000). However, despite *in situ* and freshly isolated chondrocytes not exhibiting RVI responses, treatment with Latrunculin B, an actin capping agent, induced a bumetanide-sensitive RVI response, and increased the speed of RVD in freshly isolated chondrocytes (Kerrigan *et al*., 2006). Taken together, it is suggested that actin is involved in volume regulation and mechanotransduction mechanisms (Grodzinsky *et al*., 2000, Guilak, 1995, Wang *et al*., 1993).

Conversely, RVD has been observed in both freshly isolated and *in situ* chondrocytes (Bush and Hall, 2001a). It is of note that the disruption of RVD may have a significance in the onset of OA, as the characteristic decrease of PGs and thus decreased osmolarity induce cell swelling (Hall, 1995, Wilkins *et al*., 2000). However, RVD mechanisms in chondrocytes are currently poorly understood, with an, as yet undefined, highly

permeable, low selectivity osmolyte channel thought to be the major component of RVD (Hall and Bush, 2001b, Hall *et al*., 1996b, Hall *et al*., 1996a). Cell swelling, resultant from hypo-osmotic challenge, is observed to induce an efflux of K^+ (Hall *et al*., 1996b), Cl^- (Yellowley *et al*., 2002, Yellowley *et al*., 2000) and taurine (Hall and Bush, 2001a, Hall, 1995). In other cell types, including erythrocytes, this K^+ and Cl^- efflux is mediated by the K^+-Cl^- co-transporter (KCC; (O'Neill, 1999), however, KCC has been previously observed to play no role in volume sensitive K^+ efflux (Hall *et al*., 1996b). Within chondrocytes, taurine, K^+ and Cl^- efflux induced in RVD is inhibited by tamoxifen, an inhibitor of volume-sensitive organic osmolyte anion channels (VSOAC; (Hall, 1995), thus suggesting the unidentified RVD osmolyte channel is similar to VSOAC (*Fig. 1.6*).

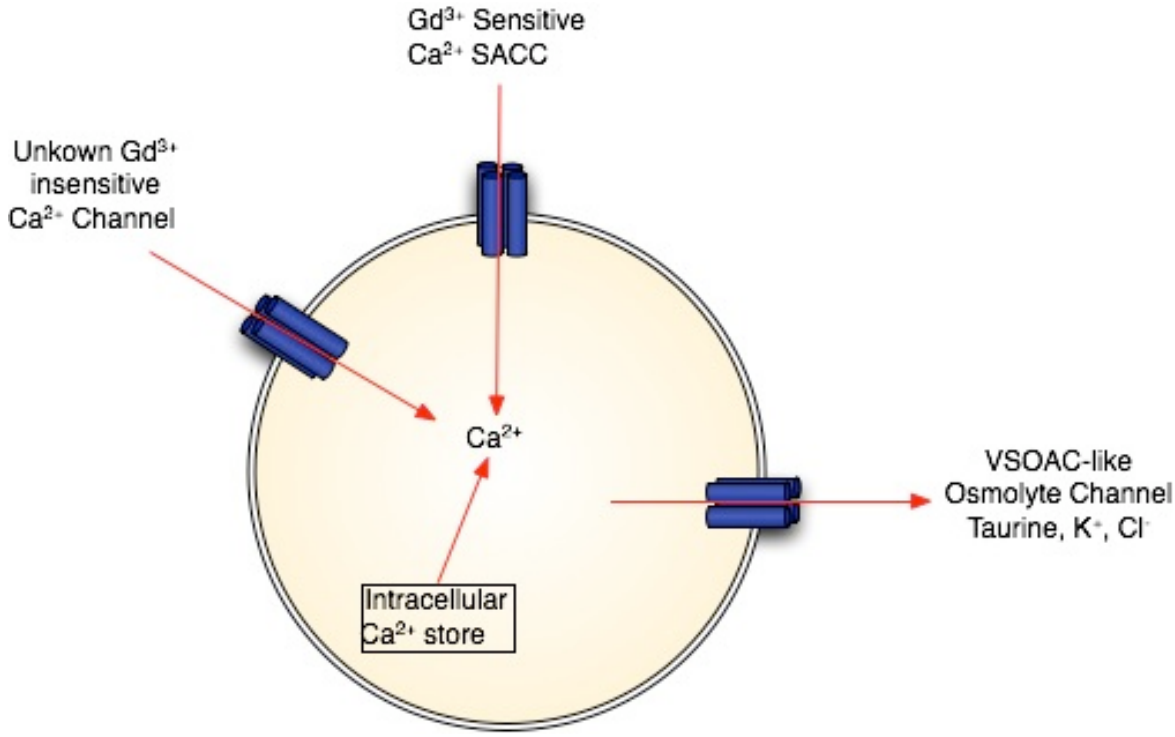


Figure 1.6: Representative diagram of the theorised regulatory volume decrease mechanism

Stimulation of regulatory volume decrease (RVD) by hypo-osmotic challenge induces an intracellular calcium ($[Ca^{2+}]_i$) rise, mediated by stretch-activated cation channel (SACC), unknown Ca^{2+} channel and release from intracellular Ca^{2+} stores. Potassium (K^+) and chloride (Cl^-) in addition to taurine are released from the chondrocyte during RVD by a tamoxifen sensitive osmolyte channel, similar to volume-sensitive organic osmolyte anion channels (Hall, 1995, Hall and Bush, 2001b, Hall et al., 1996a, Hall et al., 1996b, Kerrigan and Hall, 2007, Kerrigan and Hall, 2008a).

1.6 Mechanical Trauma and Tissue Inflammation

Following an initial mechanical impact to the tissue surrounding the knee joint, polymorphonuclear granulocytes (PMN's) followed by monocytes which differentiate into macrophages, will traffic to the site of insult (Cassatella, 1995). Mast cells and macrophages present within tissue degranulate following impact resulting in an "inflammatory milieu" comprising of mediators including cytokines/chemokines (e.g. Interleukin (IL-) 1β, 6 and 8). These mediators have the ability to influence the biological activities of responsive cells, leading to an up-regulation of adhesion molecules on the endothelium (e.g. ICAM 1) and also to L-selectin (CD62L) shedding (from PMN cell surface) thus initiating the "rolling", adhesion and subsequent emigration of leukocytes into tissue (Walzog and Gaehtgens, 2000, Lawrence and Gilroy, 2007). Following the release of these cytokines, the infiltrating leukocytes (PMN's) are no longer required and if left unchecked will lead to tissue damage due to the release of an array of toxic mediators designed to combat infection, such as hydrolytic and proteolytic enzymes and ROS. The removal of these PMNs is termed the "Resolution of Inflammation". Soon after infiltrating the tissue, the PMNs promote the production of lipoxins which initiate the resolution phase (Serhan et al., 2007). The cells undergo apoptosis, induced by the synthesis of resolvins and protectins, and express surface molecules which result in the release of anti-inflammatory "pro-resolving" cytokines including IL-10 from macrophages (Lawrence and Gilroy, 2007). The PMNs are subsequently phagocytosed by macrophages (Serhan et al., 2007).

1.6.1 The Link Between Mechanical Trauma, the Inflammatory Response and Cartilage Integrity

Cartilage metabolism is sensitive to cytokines whereby IL-1β, TNF-α and IL-17 suppress proteoglycan synthesis, change the type of collagen produced and up-regulate enzymes that breakdown cartilage (Rowan and Young, 2007, Westacott and Sharif, 1996). IL-10 and IL-13 (anti-inflammatory cytokines) are able to down regulate the effects of IL-1, TNFα and IL-17, thus reducing cartilage damage (van den Berg, 2000).

1.7 Chondrocyte Death

A common feature of OA is a decrease in the number of viable chondrocytes. Cell death occurs via several pathways, notably apoptosis and/or necrosis.

1.7.1 Apoptosis

Apoptotic cell death is the controlled breakdown of cells via a cell intrinsic mechanism, often referred to as 'programmed cell death' (Duprez *et al*., 2009). Two main protein families are involved in apoptotic signalling pathways, BCl-2 proteins which are responsible for mitochondrial integrity (Youle and Strasser, 2008), and cysteinyl aspartate-specific proteases (Caspases) which moderate the execution phase of apoptosis (Fuentes-Prior and Salvesen, 2004).

Caspases have 2 sub-families; initiators (Caspase-2, -8, -9, -10) and executioners (Caspase-3, -6, -7). Initiator Caspases are present as inactive monomers which recruited and activated by oligomerisation (Salvesen and Riedl, 2007). Caspase-mediated apoptosis is activated by intrinsic or extrinsic pathways (*Fig. 1.7*).

The intrinsic pathway is stimulated by factors such as DNA damage or cytotoxic insults and acts through the mitochondria. The mitochondrial apoptotic pathway is regulated by the BCl-2 protein family, whereby anti-apoptotic BCl-2 proteins inhibit pro-apoptotic BCl-2 proteins (Bax and Bak) from mitochondrial damage (Youle and Strasser, 2008). Upon stimulation of the intrinsic pathway, Bax and Bak inhibition is removed leading to their oligomerisation and subsequent formation of channels through which cytochrome c (cyt c) is released (Duprez *et al*., 2009). Cyt c associates with Apaf-1 and ATP, thus recruiting and activating procaspase-9 (also known as apoptosome) which in turn activates caspase-3, -6 and 7 and initiates apoptotic death (Riedl and Salvesen, 2007).

The extrinsic pathway is initiated by the stimulation of 'death receptors', of which the majority are from the tumor necrosis factor receptor (TNFR)

family. Stimulation of these receptors can induce proliferation and differentiation in addition to cell death. In order for apoptosis to be resultant, a death-inducing signalling complex (DISC) is formed, of which a Fas-associated death domain (FADD) is a component. FADD recruits and activates Caspase-8 and/or Caspase-10 which subsequently activate the executioner Caspases (Peter and Krammer, 2003). Additionally, Caspase-8 mediates the cleavage of the anti-apoptotic BCL-2 protein Bid, thus activating the intrinsic mitochondrial pathway (Duprez *et al*., 2009).

An indicative feature of apoptosis is apoptotic volume decrease (AVD), whereby cells are observed to under-go shrinkage which facilitates the breakdown of the cell into smaller, apoptotic bodies, aiding their eventual engulfment (Bortner and Cidlowski, 2002). Whilst previously thought to be a passive consequence of apoptosis, AVD is know noted to play a more active role within the apoptotic process, regulating activity of apoptotic caspases (Bortner *et al*., 1997, Hughes *et al*., 1997, Dallaporta *et al*., 1998). Whilst the mechanisms of AVD are not yet elucidated, an efflux of potassium ions is thought to be a key feature (Yu *et al*., 2001, Gomez-Angelats *et al*., 2000, Bortner and Cidlowski, 2001, Mann *et al*., 2001).

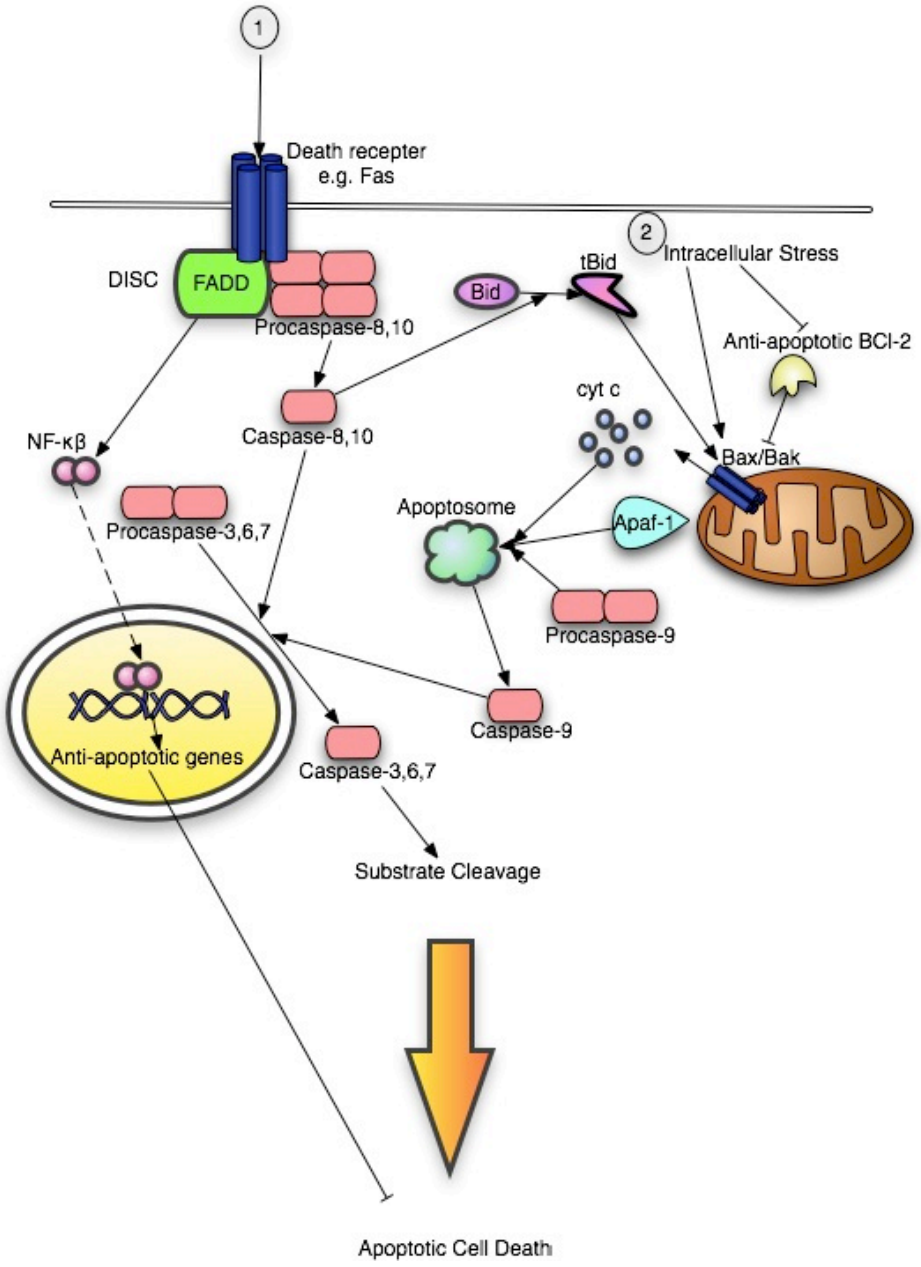


Figure 1.7: Schematic representation of extrinsic and intrinsic apoptotic signalling

Stimulation of 'death receptors' (e.g. Fas) on the cell membrane initiates the extrinsic signalling pathway (1). Upon stimulation, a death-inducing signalling complex (DISC) is formed and nuclear factor (NF)$\kappa\beta$ activated. Fas-associated death domain (FADD) is a main DISC component which recruits and activates Caspase-8 and-10 which subsequently activate the executioner Caspases-3,6,7. Additionally, Caspase-8 mediates the cleavage of the anti-apoptotic BCL-2 protein Bid, thus activating the intrinsic mitochondrial pathway (2). Intrinsic cell stress activates Bax/Bac, inducing cytochrome c (cyt c) release from the mitochondria. Subsequent formation of the apoptosome results in the activation of Caspase-9 and thus the executioner Caspases (Duprez et al., 2009, Peter and Krammer, 2003, Riedl and Salvesen, 2007, Youle and Strasser, 2008). Adapted from (Duprez et al., 2009).

1.7.2 Necrosis

Necrosis is typically thought of as 'accidental' and 'uncontrolled' cell death. It is characterised by cytoplasmic and organelle swelling leading to loss of membrane integrity and release of cell contents into the extracellular environment (Duprez *et al*., 2009). Physical damage to the cell, for example as a result of mechanical insult, results in cell death via necrosis. However, more recent studies have demonstrated the activation of necrosis via TNFR activation when in the presence of Caspase inhibition, indicating necrosis may also be a cell-mediated form of cell death and thus termed necroptosis (Degterev *et al*., 2005). Evidence suggests this pathway to be mediated by receptor interacting protein (RIP)1 (Festjens *et al*., 2007), usually cleaved by Caspase-8 and subsequently suppressing necrotic activity and promoting apoptotic (Lin *et al*., 1999, Feng *et al*., 2007). Necrosis (and necroptosis) are executed by a variety of mediators, including reactive oxygen species (ROS), Ca^{2+}, calpains, cathepsins, phospholipases and ceramide (Vanlangenakker *et al*., 2008).

1.7.3 Chondrocyte Death and Osteoarthritis

It is well documented that whilst apoptosis occurs in OA, both its relative contribution to the pathogenesis of the disease, and its cause(s) within OA are unclear (Aigner and Kim, 2002). Possible stimuli of current interest include; nitric oxide (NO), TNF-α, IL-1 and Fas receptor activation and mechanical stress (*Fig. 1.8*; (Aigner and Kim, 2002).

NO has long thought to be important in the pathogenesis of OA due to the large amount produced by OA cartilage, thus it's role in inducing chondrocyte apoptosis is unsurprising (Pelletier *et al*., 2001). Recently, it has been observed that NO-induced apoptosis is blocked by inhibitors of Caspase-3 and -9, implicating the mitochondrial pathway in the mechanism of NO-induced apoptosis (Miwa *et al*., 2000). Additionally, NO induces cyclooxygenase 2 (COX-2) expression and thus prostaglandin E_2 (PGE_2) release, as observed in human OA chondrocytes. The role of PGE_2 in initiating apoptosis however, is currently unclear, as PGE_2 stimulation has been reported to induce apoptosis in healthy bovine cartilage (Miwa *et al*.,

2000), but a separate study noted that PGE_2 simply sensitised chondrocytes to NO-induced apoptosis in human OA tissue (Notoya *et al*., 2000). It is possible the action of PGE_2 in apoptosis is species or disease state dependent.

TNF-α and IL-1 are known to be important mediators in OA, inducing the production of ceramide. Ceramide, has been reported to both increase expression of matrix metalloproteinases (MMPs; Sabatini *et al*., 2000) and induce apoptosis (Notoya *et al*., 2000), although it is unclear if these actions are concurrent or alternatives dependent on cell differentiation or activation as they are yet to be reported together. Whilst IL-1 stimulation alone has not been observed to induce apoptosis (Blanco *et al*., 1995), TNF-α stimulation has resulted in apoptosis in chick embryonic chondrocytes (Aizawa *et al*., 2001), but conversely, no apoptosis was induced in human OA chondrocytes (Kuhn and Lotz, 2001). Interestingly, combination stimulation of chondrocytes with TNF-α and proteasome inhibitor MG132 initiated apoptosis in human OA chondrocytes (Aigner and Kim, 2002). As TNFR is known to induce NF-$\kappa\beta$ activation, a recognised protective mechanism (Van Antwerp *et al*., 1996), and MG132 has been noted to inhibit this activation (Aigner and Kim, 2002), it can be hypothesised that NF-$\kappa\beta$ has a role in chondrocyte survival.

Fas receptors have been reported in chondrocytes, thus suggesting a role for Fas-mediated apoptosis, however the observation of this mechanism in chondrocytes is controversial (Hashimoto *et al*., 1998, Lisignoli *et al*., 2001, Masuko-Hongo *et al*., 2000). Fas receptor agonists have not been seen to induce apoptosis (Lai *et al*., 2001), however it has been noted that they induce DNA fragmentation, an indicator of apoptosis (Aigner and Kim, 2002). Again, co-stimulation with MG132 resulted in apoptotic death being observed, further supporting the importance of NF-$\kappa\beta$ in chondrocyte protection (Aigner and Kim, 2002).

It is acknowledged that cell attachment is required for cell function and survival in most cell types, ECM degradation and alterations, such as observed in OA and as a result of mechanical stress, induce an anchorage

disturbance (Poole *et al*., 1991, Hambach *et al*., 1998). Mechanical stress is known to induce apoptosis from pressures as low as 4.5 MPa (Loening *et al*., 2000), which has been reported as caspase dependent apoptosis (D'Lima *et al*., 2001b). It is not yet clear whether apoptosis precedes ECM destruction post mechanical stress, or if apoptosis and ECM degradation act as a destructive cycle. Additionally, mechanical stress has been observed to induce biphasic chondrocyte death, thought to be by both necrosis and apoptosis in bovine samples (Tew *et al*., 2000). Apoptosis has been reported to increase by 30 % by 96 h post mechanical load, though no significant apoptosis was noted in the first 6 h, suggesting initial chondrocyte death induced by mechanical load to be necrotic (D'Lima *et al*., 2001a).

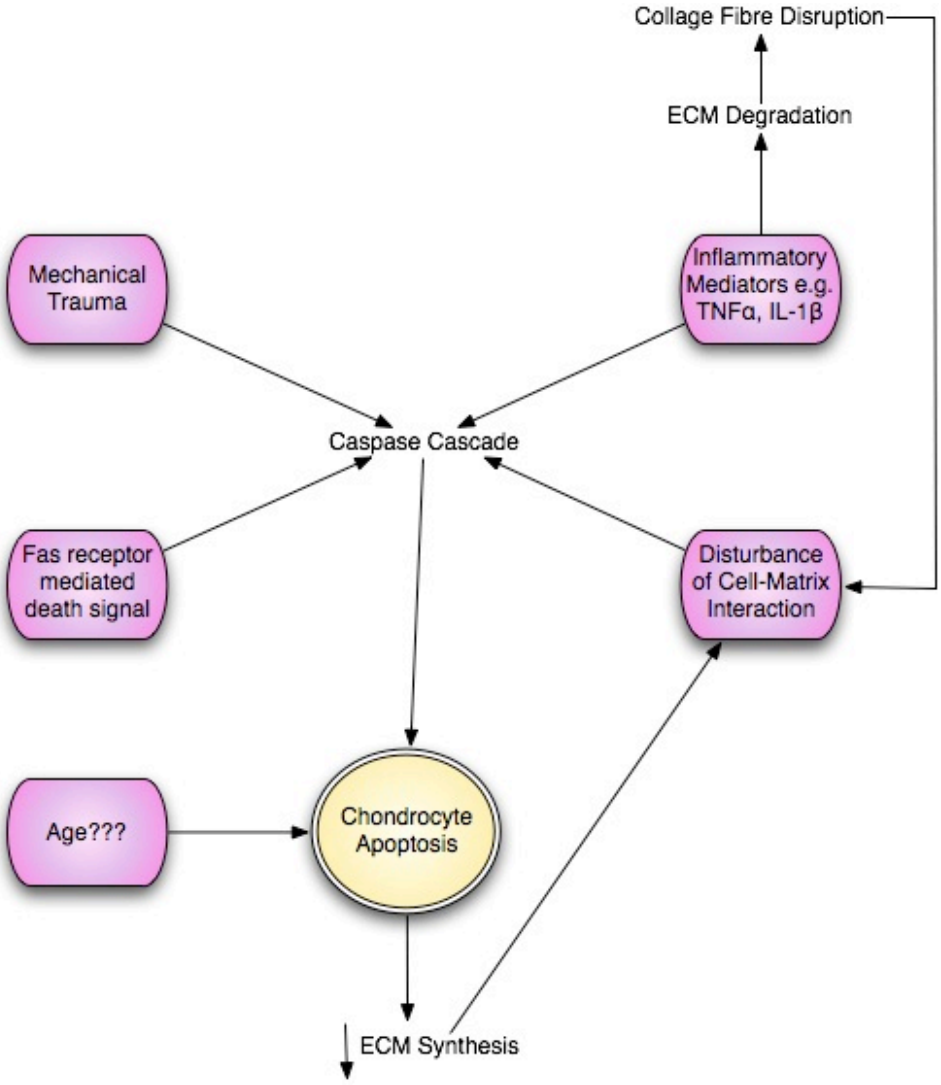


Figure 1.8: Schematic diagram of the factors leading to chondrocyte apoptosis in osteoarthritis

Inflammatory mediators such as nitric oxide (NO), tumor necrosis factor (TNF) α, ceramide and IL-1 are noted to induce chondrocyte apoptosis via the mitochondrial pathway, in addition to inducing extracellular matrix (ECM) degradation. Resultant disturbance of the ECM is additionally observed to induce apoptosis. The presence of Fas receptor in chondrocytes indicates a role for Fas-mediated apoptosis and mechanical trauma is known to induce load dependent apoptosis. The role of age in osteoarthritic chondrocyte apoptosis is not yet elucidated (Aigner and Kim, 2002, Aizawa et al., 2001, Blanco et al., 1995, D'Lima et al., 2001a, Hashimoto et al., 1998, Kuhn and Lotz, 2001, Lai et al., 2001, Loening et al., 2000, Miwa et al., 2000, Pelletier et al., 2001).

1.7.4 Chondrocyte Senescence and Osteoarthritis

Another theory for the loss of chondrocyte viability in OA is that of senescence. Cell senescence classically refers to the loss of ability of a cell to further divide and is associated with shortened telomeres; an area of repetitive DNA at the end of the chromosome (Hayflick, 1984). The shortened telomeres result progressively from repeated cell division, thus cell senescence is normally seen in older cells. This type of replicative induced senescence is commonly termed intrinsic senescence. However, in post-mitotic or quiescent cells such as neurones and chondrocytes intrinsic senescence is unlikely, thus another form of senescence has been suggested. Extrinsic senescence has been found to result from a range of stimuli including oxidative damage and inflammation, the former of which has been shown to result in telomere shortening as seen in intrinsic senescence (Itahana *et al*., 2004).

Senescent cells have been found to have an altered metabolic activity and protein expression, including an increased production of cytokines, growth factors and matrix metalloproteinases (MMPs). This is termed the senescent secretory phenotype (Campisi and d'Adda di Fagagna, 2007).

Chondrocytes from older donors have been shown *in vitro* to exhibit increased production of cytokines and MMPs, as well as increased reactive oxygen species (ROS) and indications of oxidative damage, all of which are indicative of the senescent secretory phenotype (Del Carlo and Loeser, 2002). As chondrocytes very rarely, if at all, divide it is most likely these changes are due to extrinsic senescence, which chondrocytes are thought to have an increased susceptibility due to their long lifetime. There has been evidence of telomere shortening in chondrocytes exhibiting the senescent secretory phenotype, but this has been shown to be a result of DNA damage from increased ROS (Kurz *et al*., 2005). Additionally, older *in vitro* bovine chondrocytes have been observed to produce a less functional extracellular matrix (ECM) than younger animals, this again is indicative of the senescent secretory phenotype (Tran-Khanh *et al*., 2005).

Oxidative stress is thought to play a major role in chondrocyte senescence and, thus, in the link between ageing and osteoarthritis development, as it has been observed *in vitro* that the addition of ROS induces the senescent secretory phenotype (Dai *et al*., 2006). Both aging and mechanical stress have been shown to result in increased ROS which leads to oxidative stress and thus senescence. The changes resulting from the senescent secretory phenotype in chondrocytes as a result of increased ROS from ageing and mechanical stress, all result in decreased ECM turnover and thus weakened tissue. This is a major risk factor for osteoarthritis.

1.8 Exercise, Joint Injury and Osteoarthritis

Sport/exercise participation is acknowledged to be of benefit to general health, however, it is widely acknowledged that such participation increases risk of injury (Saxon *et al*., 1999). There is also concern that excessive or prolonged participation in sport can increase risk of the development of OA later in life (Saxon *et al*., 1999). Two possible mechanisms behind the perceived increased OA risk are postulated; (1) joint injury and the resultant damage are known to increase OA risk, whereby the injury can trigger chronic cartilage remodelling or induce abnormal loading patterns (Hoffman, 1993), and (2) continuous stress placed on the joints by long-term and prolonged exercise training may result in microtrauma and degeneration (Moskowitz, 1984), thus increasing the risk of OA development. Indeed injury, especially lower limb injury, is prevalent amongst athletes, with 78.9 % of runners experiencing lower limb injury within 7 days post marathon, with the knee being the most injured site (Satterthwaite *et al*., 1996), and 2 % of all visits to accident and emergency centres in the UK in 2010 were due to sport injury. Given that the lower limb experiences forces equivalent to 2-3 times bodyweight per foot strike (Bobbert *et al*., 1991, Chan and Rudins, 1994, Dufek and Bates, 1990, Farley and Gonzalez, 1996, James *et al*., 1978), and that the average endurance runner strikes the ground 600 times per km (Milner *et al*., 2006, Pohl *et al*., 2009), this is unsurprising.

Increased joint force is noted to increase both injury and OA risk, whereby high impact sports, such as running, induce greater prevalence (Kujala *et al*., 1994, Lane, 1995, Lindberg *et al*., 1993, Saxon *et al*., 1999, Spector *et al*., 1996). Thus, there is some interest in determining means to reduce joint force without damaging performance. An area of interest is foot strike patterns, whereby the order in which the foot strikes the ground whilst running are known to vary. Two distinct styles are in evidence, 'pose' running and heelstrike running. Pose running is characterised by a mid-forefoot strike pattern, whereby the ball of the foot strikes the ground first. This style is claimed to produce better speed and thus sprinters tend to favour pose running (Hasegawa *et al*., 2007, Lieberman *et al*., 2010).

Conversely, heelstrike running involves striking the ground with the heel before the rest of the foot and is claimed to produce greater stride length, endurance runners are reported to favour heelstriking (Hasegawa *et al*., 2007, Lieberman *et al*., 2010). As yet, no definitive reports of which foot strike pattern is most effective at injury risk or force reduction, although there is evidence to suggest that heelstriking increases the force the lower limb joints are subjected to (Sol, 2001).

The increased risk in joint damage and OA development has lead to a market for products claiming to maintain 'healthy' joints. 46 % of the UK are reported to have tried such products, and approximately £450 million is spent on purchasing these products per year (Thomas *et al*., 2001, Thomas and Coleman, 2004). A few common products used include chondroitin sulphate, glucosamine sulphate, fish oil, ginger, evening primrose oil, devil's claw and flaxseed oil, with chondroitin and glucosamine sulphate (either separately or in combination) the most common (ARC, 2009). Despite the prevalence of these products, very little is known about how they work, if they work or what dosages may be needed.

1.9 Aims

The studies reported in the present thesis aim to investigate the relevance of changes in chondrocyte volume, actin and inflammation upon mechanical impact, to cell viability, and further study the osmotic sensitivity of these mechanisms. Additionally, nutriceuticals and pharmaceuticals were studied to isolate possible chondroprotective mechanisms. Furthermore, *in vivo* studies aimed to elucidate diurnal links between joint forces and inflammation.

Chapter 2: Materials & Methods

2.1 Materials

2.1.1 Tissue Culture

Name	Company	Product Code	Working Concentration
Dulbecco's Modified Eagle Medium (DMEM)	Sigma-Aldrich, Poole, UK	D6171	N/A
Penicillin / Streptomycin Solution	Sigma-Aldrich, Poole, UK	P0781	1%
Sodium Chloride	Fisher Scientific, Leicestershire, UK	S13160/65	Varied
Roswell Park Memorial Institute Medium (RPMI)	Hyclone, Utah, USA	SH30096.01	N/A
L-Glutamine	Sigma-Aldrich, Poole, UK	C93126	2mM
Foetal Calf Serum	Invitrogen, Paisley UK	16170-078	10%
Trypsin-EDTA	Invitrogen, Paisley UK	25300	0.05%

Table 2.1: Tissue culture reagents.

2.1.2 Cytoskeleton Studies

Name	Company	Product Code	Working Concentration
Bovine Serum Albumin	Sigma-Aldrich, Poole, UK	A2153	1%
Paraformaldehyde	Sigma-Aldrich, Poole, UK	533998	4%
Phosphate Buffer Saline	Oxoid Ltd, Hertfordshire, UK	BR0014G	N/A
Triton X100	Fisher Scientific, Leicestershire, UK	ZT3751	0.5%
Ammonium Chloride	Sigma-Aldrich, Poole, UK	A0171	50mM
Glycine	Fisher Scientific, Leicestershire, UK	BPE381	100mM
Tween 20	Fisher Scientific, Leicestershire, UK	BPE337	0.05%

Table 2.2: Reagents used to study the actin cytoskeleton.

2.1.3 ELISA Reagents

Name	Company	Product Code	Working Concentration
human Immunoassay kit	R&D Systems, Abingdon, UK	DY201/DY208/ DY217B/DY279	N/A
LTB_4 Assay Kit	R&D Systems, Abingdon, UK	KGE006B	N/A
Phosphate Buffer Saline	Oxoid Ltd, Hertfordshire, UK	BR0014G	N/A
Tween 20	Fisher Scientific, Leicestershire, UK	BPE337	0.05%
Bovine Serum Albumin	Sigma-Aldrich, Poole, UK	A2153	0.1%, 1%
Hydrogen Peroxide	Fisher Scientific, Leicestershire, UK	H1550	0.02%
Tetramethylbenzidine	Sigma-Aldrich, Poole, UK	T5525	0.1mg/ml
Dimethyl Sulphoxide	Fisher Scientific, Leicestershire, UK	D4121	1%
Sulphuric Acid	Fisher Scientific, Leicestershire, UK	J/8410/17	2M
Phosphate Citrate Buffer	Sigma-Aldrich, Poole, UK	P4809	0.05M

Table 2.3: Reagents used during ELISA

2.1.4 Fluorophores

Name	Company	Product Code	Solvent	Working Concentration
Calcein AM	Anaspec Inc, Freemont, USA	89201	DMSO	5μM
Alexa 488 (conjugated to phalloidin)	Invitrogen, Paisley, UK	A12379	PBS	5μl/ml
Propidium Iodide	Invitrogen, Paisley, UK	P1304MP	DMSO	1μM

Table 2.4: Fluorophores used for confocal laser scanning microscopy.

2.1.5 Pharmacological Reagents

Name	Company	Product Code	Working Concentration	Comment	References
REV5901	Sigma-Aldrich, Poole, UK	R5523	50 μM	Inhibitor of RVD and Lipoxygenase	(Bush and Hall, 2001a, Musser *et al*., 1987, Van Inwegen *et al*., 1987)
U73122	Sigma-Aldrich, Poole, UK	U6756	100 μM	PLCβ3 inhibitor	(Hou *et al*., 2004)
Rottlerin	Sigma-Aldrich, Poole, UK	R5648	100 μM	PKC inhibitor	(Gschwendt *et al*., 1994)
Dimethyl Sulphoxide	Fisher Scientific, Leicestershire, UK	D4121	0.1 %	Vehicle of REV5901	-
Ethylene glycol tetraacetic acid (EGTA)	Sigma-Aldrich, Poole, UK	E0396	2 mM	Ca^{2+} Chelator	(Kerrigan and Hall, 2008)
Tamoxifen	Fisher Scientific, Leicestershire, UK	32932-0010	10 μM	Inhibitor of PKC and calmodulin	(Wiseman, 1994, Shinmei *et al*., 1988)
Dexamethasone	Sigma-Aldrich, Poole, UK	D4902	1 μM	Anti-inflammatory glucocorticoid	(Shinmei *et al*., 1988)
Curcumin	Sigma-Aldrich, Poole, UK	C1386	50 μM	Inhibitor of MCP-1	(Sharma *et al*., 2005)
Chondroitin Sulphate	Organika, Richmond, Canada	N/A	0.3 mg/ml	Commonly used supplement for joint care	(ARC, 2009)
Glucosamine Sulphate	Morrisons, Bradford, UK	N/A	0.1 mg/ml	Commonly used supplement for joint care	(ARC, 2009)
Human IL-1β	Sigma-Aldrich, Poole, UK	I9401	200 pg/ml/g	Pro-inflammatory cytokine	(Shinmei *et al*., 1988)
Jasplakinolide	Invitrogen, Paisley, UK	J7473	5 μM	Stabilises actin filaments	(Bubb *et al*., 1994)
Latrunculin B	Sigma-Aldrich, Poole, UK	L5288	10 μM	Inhibits actin polymerisation	(Coue *et al*., 1987)

Table 2.5: Pharmacological agents and nutritional supplements used

2.1.6 Culture Media and Experimental Salines

Bovine tissue samples were cultured using Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1 % penicillin/streptomycin solution (pen/strep) under aseptic conditions. DMEM with an osmolarity of 280 mOsm/kg H_2O (mOsm) was termed isotonic as synovial osmolarity has been previously observed to be 280-300 mOsm (Hall et al., 1996a). To investigate the effects of a hyperosmotic challenge, the osmolarity of DMEM was adjusted to 380 mOsm by the addition of 3.14 mg/ml of filter-sterilised sodium chloride (NaCl) using a 2 μm filter. Conversely, to investigate the effects of a hypo-osmotic challenge, the osmolarity of DMEM was adjusted to 140 mOsm by the addition of filter-sterilised distilled water (ddH_20). The osmolarity was measured using Vapro™ vapour pressure osmometer and accurate to 0.1 mOsm.

For experiments that required the removal of extracellular calcium ($[Ca^{2+}]_e$), basic physiological saline (BPS) containing; HEPES (15 mM), glucose (10 mM), potassium chloride (KCl; 5 mM), magnesium chloride ($MgCl_2$; 1 mM) and either ethylene glycol tetraacetic acid (EGTA; 2 mM) or calcium chloride ($CaCl_2$; 1 mM) as a paired control. The pH was measured using Fisher Scientific pH meter and adjusted to 7.40 ± 0.05 using sodium hydroxide (NaOH), and the osmolarity measured using Vapro™ vapour pressure osmometer and adjusted by the addition of NaCl.

2.2 Bovine Dissection

Bovine metacarpal and metatarsal phalangeal joints from freshly slaughtered 18-21 month old females (obtained from a local abattoir with permission, used within 2 days), were de-skinned and de-hoofed to expose the joint capsule (Hall, 1995). Under aseptic conditions the joint capsule was opened (*Fig. 2.1*) and full depth cartilage explants excised and stored in 280 mOsm/kg H_2O DMEM containing 1 % penicillin/streptomycin for up to 24 h.

2.2.1 Mononuclear Phagocyte Isolation and Culture

Upon bovine dissection synovial fluid was removed from the metacarpal/metatarsal phalangeal joint and diluted in RPMI culture media and the cells subsequently washed by centrifugation. The cells were plated in flasks, the culture resulting from each individual leg plated separately. After allowing 24 h for cell attachment and neutrophil death, the non-adhering cells were removed and the culture incubated with trypsin (Adams, 1979). The resultant supernatant was removed and discarded and the trypsin-resistant mononuclear phagocytes lifted from the flask by gentle scraping and seeded at a density of 1 x 10^4 cells/cm^2 (Dechanet *et al*., 1995). Cells were used within two weeks of isolation (Adams, 1979, Dechanet *et al*., 1995).

The mononuclear phagocytes were cultured with the supernatants taken from impacted explants 4 h post impact, or with impacted explants for 4 h and the supernatants removed and analysed for cytokines of interest (*Section 2.6*).

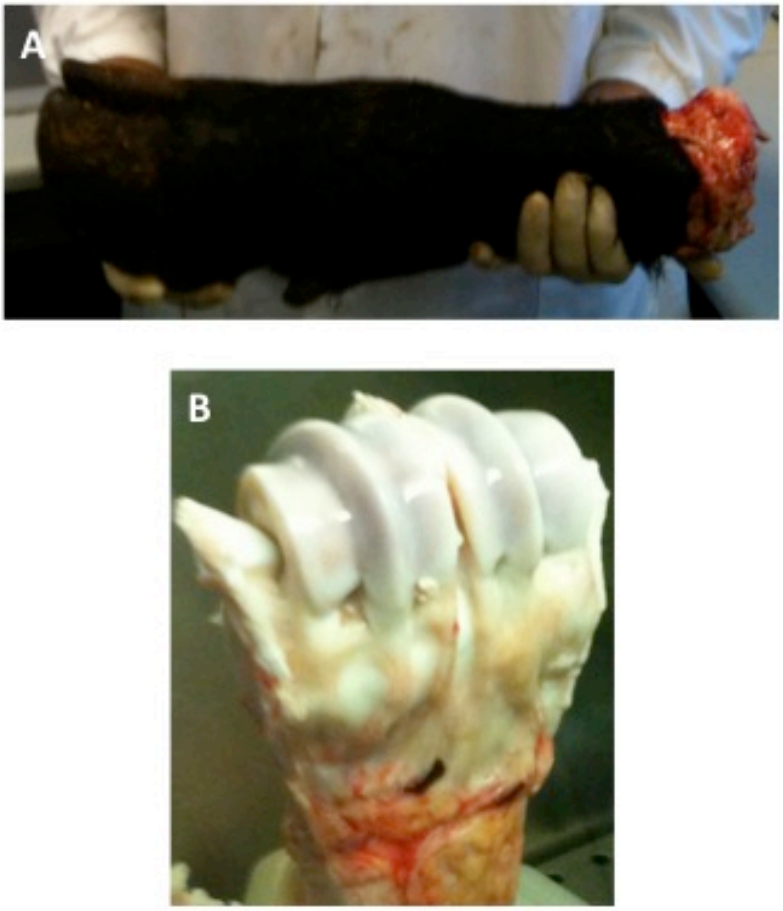


Figure 2.1: Bovine dissection of full-depth explants

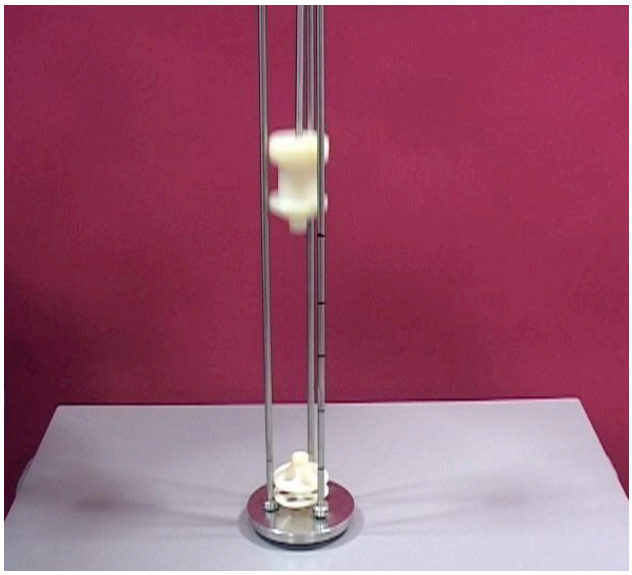
A) An undissected bovine metacarpal/metatarsal phalangeal joint. B) The apical surface of the metacarpal/metatarsal joint from which full depth explants were taken.

2.3 Impact Tower

To investigate the effects of impact trauma on articular cartilage and cell death, each individual cartilage explant was subjected to a single impact using a vertical 'drop tower' following a previously published method (*Fig. 2.2*; (Bush *et al*., 2005). Assuming linear acceleration under gravity, known mass (134 g) and height (10 cm) of the "drop", the impact force, pressure and energy were calculated.

To confirm the accuracy of these theoretical, calculated values as well as the precision of the technique, video analysis of the drop tower was carried out, measuring the exact time the load took to drop from 5 cm, 10 cm and 20 cm. It was concluded that when dropped from a height of 10 cm an impact of 1.14 N, and 6.47 Kpa was measured and that the technique was reproducible from all heights (*Table 2.6*).

Whilst these values are significantly lower than published values for human *in vivo* joint forces (oscillating between 0.2 and 4-5 MPa in the hip during the normal walking cycle; (Hodge et al., 1986), they have been optimised as an efficient magnitude for investigating cell death as a result of impact to dissected articular cartilage (*Fig. 2.3*), providing sufficient measurable cell death to study without destroying the tissue. It should also be noted that as the explants in question have neither the support of the bone or the protection of synovial fluid, as they would *in vivo*, they would not be expected to withstand the large forces they are subject to *in vivo* and thus the impact tower model is not intended as an exact model for human joint forces, but merely as a tool to investigate the effects of mechanical impact on the tissue.



	Theoretical (10cm)	Experimental (10cm)
Work (J)	0.134±0.00	0.114±0.00
Pressure (Kpa)	7.58±0.00	6.47±0.07
Force (N)	1.34±0.00	1.14±0.01

Table 2.6: Theoretical and experimental forces for the impact tower

Theoretical values were calculated using basic physics equations. Experimental values were obtained by video analysis of the impact tower being dropped from 10cm. The time taken to drop was used to calculate velocity (v = d/t) and, thus, acceleration (a=v/t). Force was then calculated from the known mass (134 g) and the acceleration (F=ma). Pressure was calculated from the force and the "drop" area (1.77 cm^2) of the impact tower (P=F/A). Work was calculated from the force and the distance (W=Fxd). N=10

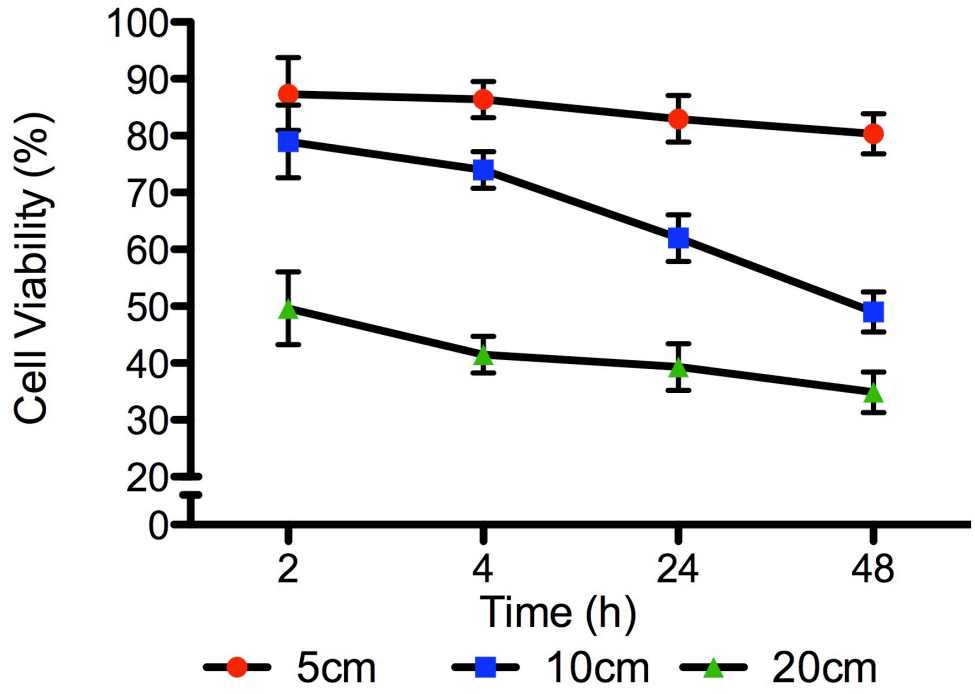


Figure 2.3: Optimisation of drop tower height

Explants were subjected to a single impact using the vertical drop tower from 5, 10 or 20 cm and analysed for cell viability using confocal laser scanning microscopy. Samples were incubated with 5 μM calcein-AM and 1 μM propidium iodide prior to visualisation to indicates viable and non-viable cells respectively. Samples were imaged at 2, 4, 24 and 48 h post impact. Viability is expressed as a percentage of the total cell number. 10cm was observed to be the most effective at inducing measurable cell death without destroying the sample. N=6 joints

2.4 Tissue Fixing and Cytoskeletal Staining

In order to investigate the effects of impact trauma on actin cytoskeletal organisation, bovine explants were fixed before impact and at 0, 2, 4, 24 & 48 h post impact in ice-cold 4 % paraformaldehyde (PFA) for 1 h at 4°C. The samples were then subsequently washed 3 times in ice-cold PBS to remove excess PFA and then quenched for 10 min in ice-cold 50 mM NH_4Cl. Subsequently the samples were permeabilsed with 0.5 % Triton X100 in PBS for 5 min on a rocker and dipped in 100 mM Glycine to stop permeablisation before being washed 3 times in ice-cold PBS and blocked with 1 % bovine serum albumin (BSA) and 0.05 % Tween-20 in PBS. After washing 3 times with ice-cold PBS the samples were labelled with Alexa 488-phalloidin (5 μl/ml) and placed on a rocker in the dark for 45 min. After incubation the samples were washed 3 times in 0.05 % Tween in PBS and visualised using CLSM.

2.5 Confocal Laser Scanning Microscopy (CLSM)

Confocal laser scanning microscopy (CLSM) is a powerful tool that permits the *in vitro* 3D imaging of interior structures within tissue (Jones *et al*., 2005, Kerrigan and Hall, 2008a). By controlling the depth of focus, CLSM allows the acquisition of individual images from multiple selected depths (termed optical sectioning), which are subsequently reconstructed resulting in three-dimensional (3D) objects. Briefly, a laser beam passes through the light source aperture and is focused onto a focal point on the surface or within the sample being imaged by the objective lens. The fluorescent light emitted from the fluorophores used to stain the sample, is collected by the objective and separated into the detection aperture by the beam splitter where it is passed through the pinhole, which blocks any light not returning from the focal point thus resulting in a sharper image. The light signal is ultimately converted into an electrical signal able to be interpreted by a computer by a photodetection device. By assembling images from successive focal planes (termed a z-stack), a 3D image of the sample can be acquired.

CLSM was utilised to investigate changes in cell viability, cell volume and the actin cytoskeleton. For imaging to determine cell viability and volume, samples were visualised at 0, 2, 4, 24 and 48 h post impact. Prior to visualisation samples were incubated for 30 min at 37°C and 5% CO_2 with 5 μM Calcein-AM and 1 μM Propidium Iodide (PI) to indicate viable and non-viable cells respectively. Viability images were acquired using a x10 objective and a z-step of 5 μm and volume images using a x63 dipping objective and a z-step of 1 μm. Calcein-AM was excited using a laser with an excitation wavelength of 488 nm and an emission passband of 510-590 nm was recorded (*Fig. 2.4A)*. PI was excited at a wavelength of 543 nm and an emission passband of 570-710 nm was recorded (*Fig. 2.4B*). For imaging to investigate the actin cytoskeleton samples were fixed and stained using Alexa 488-phalloidin as previously described (*Section 2.4*). Actin images were acquired using a x63 dipping objective and Alexa 488-phalloidin was excited using a laser with an excitation wavelength of 488 nm and a recorded passband of 535-590 nm (*Fig. 2.4C)*. All images were acquired at 0.6 Hz and the power output of each laser adjusted to ensure maximal excitation with minimal bleaching.

During the optimising of the CLSM settings, it was noted that there was the potential for bleedthrough between calcein-AM and PI, as the emission passband of each overlaps (*Fig. 2.4*). This could lead to false images when imaging samples stained with both fluorophores. In order to combat this, a sequential imaging technique was used. Sequential imaging prevents bleedthrough by exciting each fluorophore consecutively instead of simultaneously. This technique ensures the image acquired is solely due to the emitting fluorophore and not due to bleedthrough from the second fluorophore (Leica, 2006, Ramshesh and Lemasters, 2008).

All Images were acquired at a resolution of 1024x1024 pixels using an optimal pinhole radius of 1 Airy unit, which is defined as the diameter of the Airy disc for each given objective and excitation wavelength (Ramshesh and Lemasters, 2008). Briefly, the Airy Disc refers to the inner light circle of the diffraction pattern of a point light source. For the Leica Confocal

System, as used in this study, the diameter of the Airy Disc, and thus 1 Airy unit can be described as:

$$d_{Airy} = \frac{1.22\lambda}{NA} \times 3.6M$$ (Leica, 2004)

Where:

$d_{Airy} \rightarrow$ Diameter of Airy disc

$\lambda \rightarrow$ Excitation wavelength

$NA \rightarrow$ Numerical aperture of objective

$M \rightarrow$ Magnifying factor of objective

By adjusting the pinhole radius to the diameter of the Airy disc, or 1 Airy unit, light from outside the focal plane is suppressed and the signal to noise ratio is thus high, resulting in optimal acquisition.

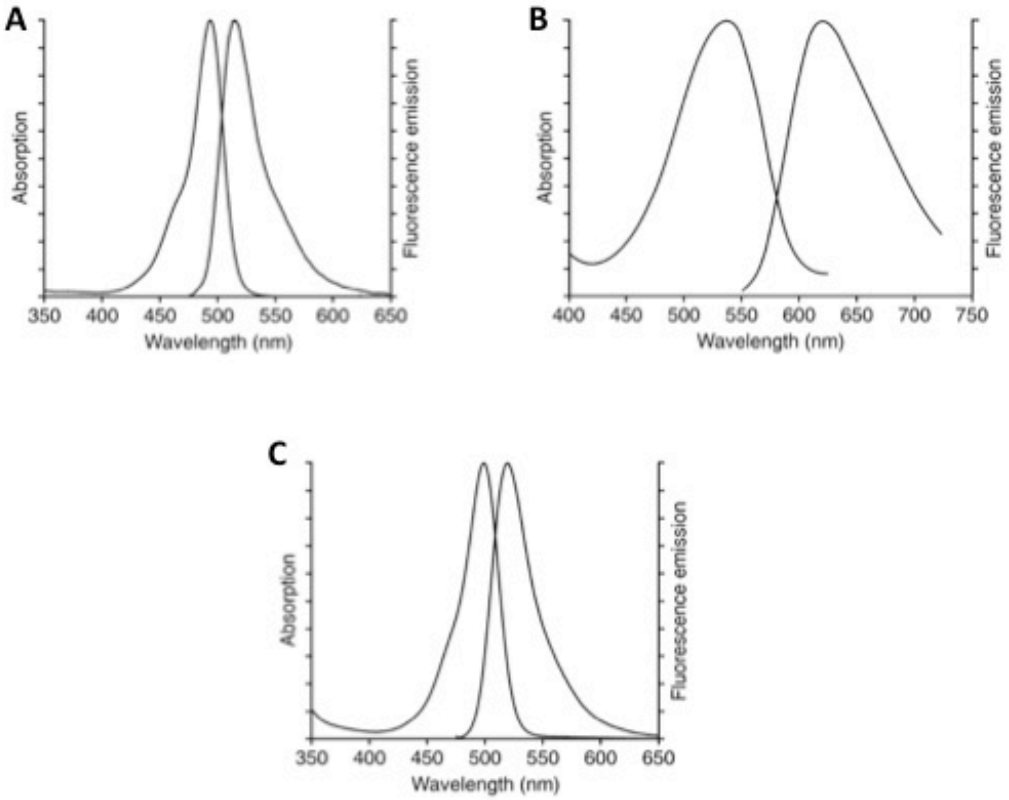


Figure 2.4: Excitation and Emission spectra for Calcein-AM, Propidium Iodide and Alexa Fluor 488 Phalloidin.

Observation of the spectra for the fluorophores revealed excitation wavelengths of 488 nm for both calcein-AM and alexa fluor 488 phalloidin, and 543 nm for propidium iodide. Optimal passband for calcein-AM was observed to be 510-590 nm and propidium iodide to be 570-710 nm. The overlapping of the passband for these two fluorophores used simultaneously indicated the need for sequential imaging. Optimal passband for alexa fluor 488 phalloidin was observed to be 535-590 nm. Adapted from Anaspec and Invitrogen product datasheets.

2.6 ELISA

Following impact, supernatants were collected and analysed for production of pro and anti-inflammatory mediators by ELISA. The levels of IL-1β, IL-10 and MCP-1 were assessed using the commercially available Duoset® human Immunoassay kits (R & D systems), additionally the levels of LTB_4 was assessed using LTB_4 Immunoassay kit (R & D systems). These quantitative colorimetric assays were conducted according to the manufacturer's protocol. Briefly, the capture antibody was pre-coated onto a high-binding 96 well microplate overnight at room temperature. The plate was subsequently washed and blocked for 1 h with 1 % BSA in PBS. It should be noted that the previously described steps were unnecessary for the LTB_4 assay as the plate is coated prior to purchase. Sample (100 µl) was added to the appropriate wells and bound to the immobilised antibody. Unbound sample and standard were removed by washing and a biotin-labelled detection antibody added for 2 h forming an analyte-antibody complex. Unbound detection antibody was removed by washing and Streptavidin-HRP added for 20 min at room temperature. Following the removal of non-bound antibodies using the washing procedure the TMB/peroxide (substrate) was added and was converted by the HRP (enzyme) to form a blue colour. The intensity of the colour indicating the concentration of the cytokine present within the sample. Following a 20 min incubation, 50 µl of 2M H_2SO_4 was added to terminate the reaction and thus turn the blue colour to a stable yellow product. The concentration of the cytokine detected was measured spectrophotometrically at 450 nm with a wavelength correction of 570 nm. The levels of cytokines present within the samples was compared to a standard curve for each cytokine ranging from (0 – 2000 pg/ml) and constructed using 2-fold serial dilution with the appropriate standard and the calibrator diluent (supplied within the kit; *Fig. 2.5*).

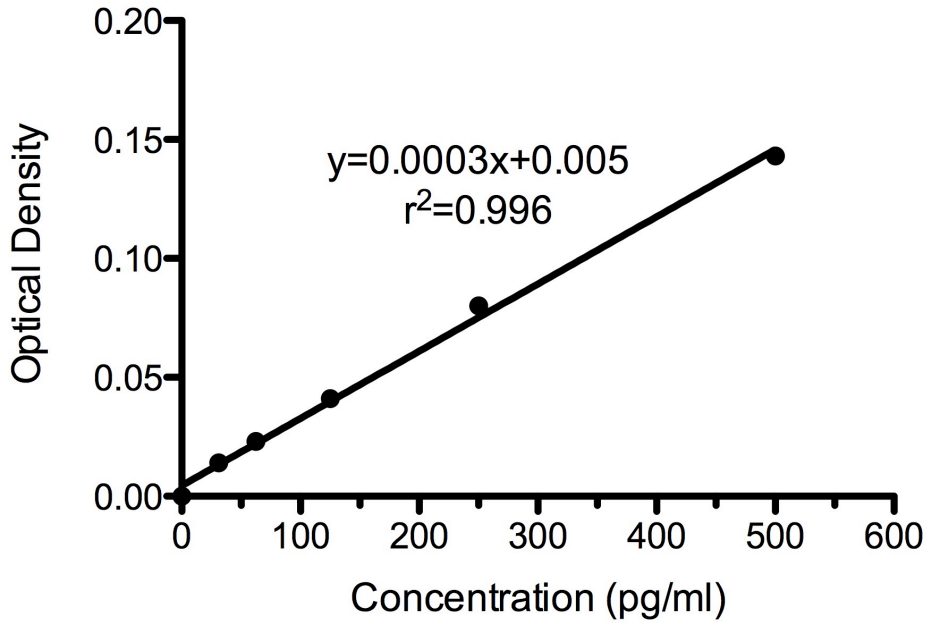


Figure 2.5 Example ELISA standard curve for IL-1β

Average optical density for triplicate standards was plotted against concentration. Concentration of experimental samples were calculated using the equation generated by the best fit curve.

2.7 Human Testing

The effects of diurnal exercise on circulating inflammatory cytokines was investigated by the recruitment of a pool of healthy male participants. Initially, in order to standardise the joint forces across the participant population and thus ensure a homogenous set of participants for cytokine analysis, the effect of physiological characteristics on the magnitude of joint loading was investigated by recruitment of a varied pool of participants and subsequent physiological analysis. By investigating the correlations between physiological characteristics and joint forces, the characteristics needing to be controlled were elucidated.

A total of 48 male and female participants were recruited by mailing list emails and poster advertising from within the University of Westminster and from London based running, cycling and triathlon clubs. After completion of a retrospective "Training and Injury Questionnaire" and "medical form" (Appendix I; modified with permission from (Vleck and Garbutt, 1998), the participants were divided into 4 groups; Sedentary Females (SF; N=10), Sedentary Males (SM; N=10), physically Active Females (AF; N=14) and physically Active Males (AM; N=14). AF and AM were defined in this study as individuals that participated in more than 3 h of training per week over the last year. Participants were analysed for physiological parameters including height, body mass, percentage body fat, leg power (as estimated by vertical jump height) and thigh circumference (Section 2.7.1) and analysed for joint forces when running barefoot (Section 2.7.2). Statistical comparison between the different groups revealed a significant difference, both between males and females, and between active and sedentary participants, indicating gender and activity level affected joint forces (Table 2.7). Additionally, correlations between both body mass and percentage body fat, as well as thigh circumference with joint forces indicated these three physiological factors all affected joint force magnitude (Table 2.8). No correlation was seen with leg power or velocity, indicating these factors did not need to be controlled. Thus it was concluded that participants for the study should be male, active, with a controlled thigh circumference, body mass and percentage body fat.

	Significance value	Significant difference?
Male vs. Female	0.00018	Yes
Active vs. Non-Active	0.02002	Yes

Table 2.7: Student T-Test analysis of *in vivo* running knee impact force differences between males and females, and actives and non-actives.

Dominant leg running knee forces were compared between males and females, and between actives and non-actives. There was a significant difference observed between the genders and activity levels indicating both these factors needed to be controlled to standardise knee force. N=48 participants

Factor vs. Knee Force	Pearson Correlation Coefficient	Significance value	Correlation?
Leg Power	0.2044	0.1886	No
Body Mass	0.6579	0.000002	Yes
Percentage Body Fat	-0.6716	0.000001	Yes
Velocity	0.0969	0.5364	No
Thigh Circumference	0.639	0.000004	Yes

Table 2.8: Pearson correlation coefficient analysis between *in vivo* running knee force and physiological characteristics.

Correlations between dominant leg running knee force and physiological parameters were investigated using the Pearson Coefficient. A significant correlation was observed when knee force was compared to body mass, percentage body fat and thigh circumference, indicating all these parameters needed to be controlled to standardise knee force. N=48 participants

2.7.1 Participant Characteristics

A total of 14 participants were recruited by mailing list emails and poster advertising from within the University of Westminster and from London based running, cycling and triathlon clubs. Participants accepted were active males, aged 20 – 39 years old, with a body mass of 65 – 75 kg, a percentage body fat of 10 – 13 % and a dominant leg thigh circumference of 48 – 53 cm. Dominant leg was determined as the leg stepped back onto when the participant was pushed backwards.

Participants were weighed and measured for height whilst barefoot. In order to ensure the participants were healthy, resting heart rate (HR) and blood pressure (BP) were taken 3 times using a Polar heart rate monitor and sphygmomanometer (A&D,Oxfordshire, UK) respectively.

Anthropometric measures were collected with the participants dressed solely in tight-fitting underwear/swimwear. Limb circumferences were measured on the thigh to standardise the participant pool as indicated by the initial testing. Lengths of the tibia and femur also collected to be used later in combination with forceplate data for calculation of the joint forces. Tibia length was measured from the tibiale mediale to the sphyrion tibiale and femur length from the trochanterion to the tibiale laterale (*Fig. 2.6*). All measurements were taken according to standard protocol using measuring calipers (Marfell-Jones, 1991).

A BODPOD (Concord, CA, USA) system was used to collect percentage body fat and lean. After weighing the participant entered the BODPOD dressed in tight-fitting underwear/swimwear with all jewellery/glasses etc removed and with hair covered in a tight cap. The BODPOD performed 3 measurements and gave an average reading for percentage body fat and percentage lean mass.

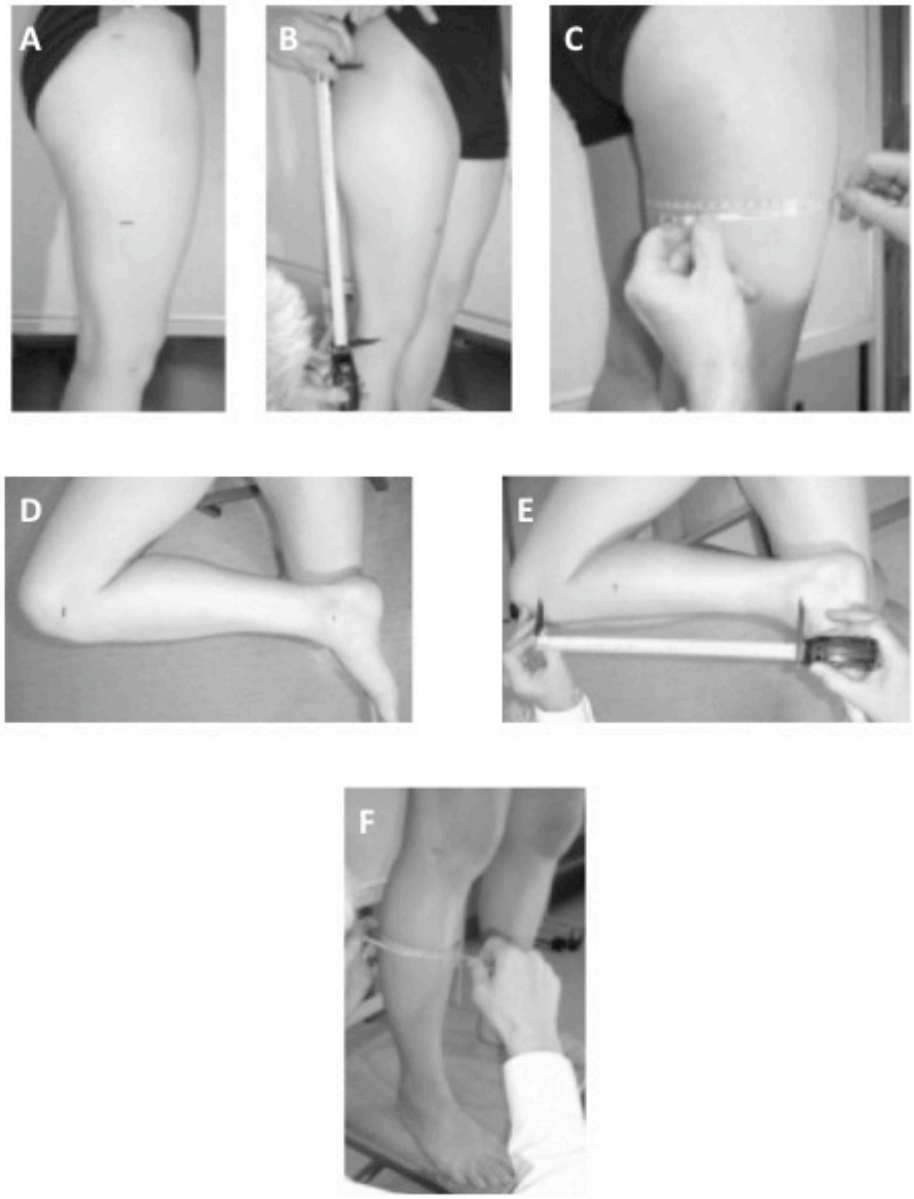


Figure 2.6 Anthropometric measurements.

A) Identification of the landmarks trochanterion, mid trochanterion-tibiale laterale and tibiale laterale. B) Measurement of thigh length, the distance between the trochanterion and tibiale laterale landmarks. C) Measurement of thigh circumference, taken at the mid trochanterion-tibiale laterale landmark. D) Identification of the landmarks tibiale mediale and sphyrion. E) Measurement of tibia length, the distance between the tibiale mediale and sphyrion landmarks. E) Measurement of calf circumference, taken at the observed widest point. Images adapted from (Marfell-Jones, 1991).

2.7.2 Mechanical Testing

Barefoot participants stood on the Forceplate (AMCube, France) with both feet and the pressure profile generated was analysed. Participants then ran across the force plate (*Fig. 2.7*) 10X times, 5X with the left foot hitting the plate and 5X with the right foot, at a pace similar to "long distance running" (13.4 $km.h^{-1}$; (Zamparo *et al*., 2001). The pressure profiles were analysed and used to generate maximum force exerted onto the mat for each foot. These values were later used to calculate joint impact forces (*Section 2.9.4*).

2.7.3 VO_2max

All participants underwent a submaximal aerobic fitness test to estimate VO_2max. The participants warmed up on a treadmill (HP Cosmos, Gaiam, UK) set a 0 % grade and a walking speed sufficient to bring the heart rate (HR) to 50 – 70 % of the individual's age-predicted maximal HR (220 - age) for 4 min. Subsequently, whilst the treadmill speed was kept the same, the incline was increased to 5 % and the subject continued to exercise for a further 4 min. HR was recorded every 5 sec and averaged for the final 30 sec of each of the last 2 min. If these two values differed by more than 5 bpm, the test was extended for an additional minute until the average HR of the last 30 sec of the final 2 min were within 5 bpm. The median of these two values was then termed the steady-state HR (SS HR) and used to estimate VO_2max using the following equation:

Estimated VO2max (ml.kg-1.min-1) = 15.1+21.8 (speed in mph) – 0.327 (SS HR in bpm) – 0.263 (speed in mph x age in years) + 0.00504 (SS HR in bpm x age in years) + 5.98 (gender; male=1, female=0)

(Ebbeling *et al*., 1991)

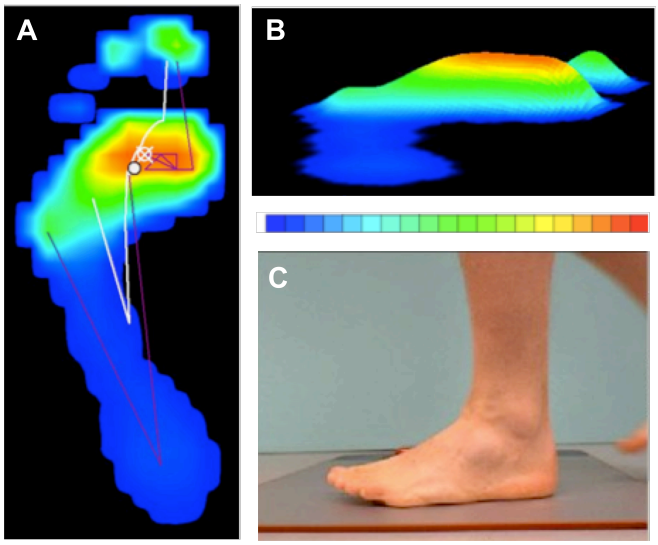


Figure 2.7: Representative Image of Gait Analysis Using Footwork Pro.

(A) Plantar elevation of ground reaction force (GRF). The red line shows force transference and the white line shows centre of mass. (B) 3D posterior elevation of GRF. (C) Barefoot running on the forceplate. Colour Scale: Represents force magnitude within the step from blue (lowest) to red (highest).

2.7.4 Diurnal Exercise

Participants underwent 30 min of treadmill exercise at an intensity of 60 % VO_2max. Each participants' running speed was calculated from their VO_2max previously measured. HR was monitored continuously through-out the session. Venous blood (6 ml) was obtained from the antecubital vein using a Vacutainer lancet and EDTA collection tube (BD Diagnostics - Preanalytical Systems) prior to, immediately post and at 2 and 4 h post exercise. Participants commenced exercise at either 8am or 6pm. For the 24 h preceding the tests participants were asked not to take part in any strenuous activity (i.e. no training sessions) or take any anti-inflammatory medications. Between blood samples participants were asked to sit and refrain from any unnecessary activity and to not consume medications or stimulants. Blood samples collected were stored at 4°C prior to centrifugation at 2500 rpm for 10 min. The blood serum was then removed and stored at -80°C until analysis. The levels of IL-1β, MCP-1 and IL-10 were assessed using the commercially available Duoset® human Immunoassay kits (R & D systems). This quantitative colorimetric assay was conducted according to the manufacturer's protocol as previously described (*Section 2.6*).

2.8 Data Analysis

2.8.1 CLSM Viability Analysis

To determine cell viability post impact, CLSM data was imported into and analysed using Imaris 7.1 (Bitplane; Netherlands). Based on the average diameter of the “live” green cells and the “dead” red cells in the explant, spot analysis was used to determine the total number of cells and the number of viable cells within the explant (*Fig. 2.8*). Viable cells were then expressed as a percentage of the total cells within the sample.

The optimal threshold for calcein-AM and PI when using spot analysis was calculated by selecting an area with a countable, known number of cells. Different threshold percentages were then applied and a linear relationship observed between threshold percentage and cell number. The correct cell number was shown to be ascertained with a threshold percentage of 20 % for cells stained with calcein-AM and 60 % for PI.

2.8.2 CLSM Volume Analysis

Analysis of cell volume changes post impact was ascertained by importing CLSM volume images into Imaris 7.1 (Bitplane; Netherlands). An “Isosurface” was added to each individual cell and the volume of the resultant object calculated (*Fig. 2.9*).

A threshold percentage of 60 % was employed, and confirmed as optimal using “bead analysis”. Briefly, glass beads of a known volume (520 μm^3) were imaged and Isosurface volume analysis applied with a spectrum of threshold percentages. A linear relationship was found between the threshold percentage and the apparent volume (*Fig. 2.9*) with the correct volume determined by line regression to be yielded at 60 % threshold.

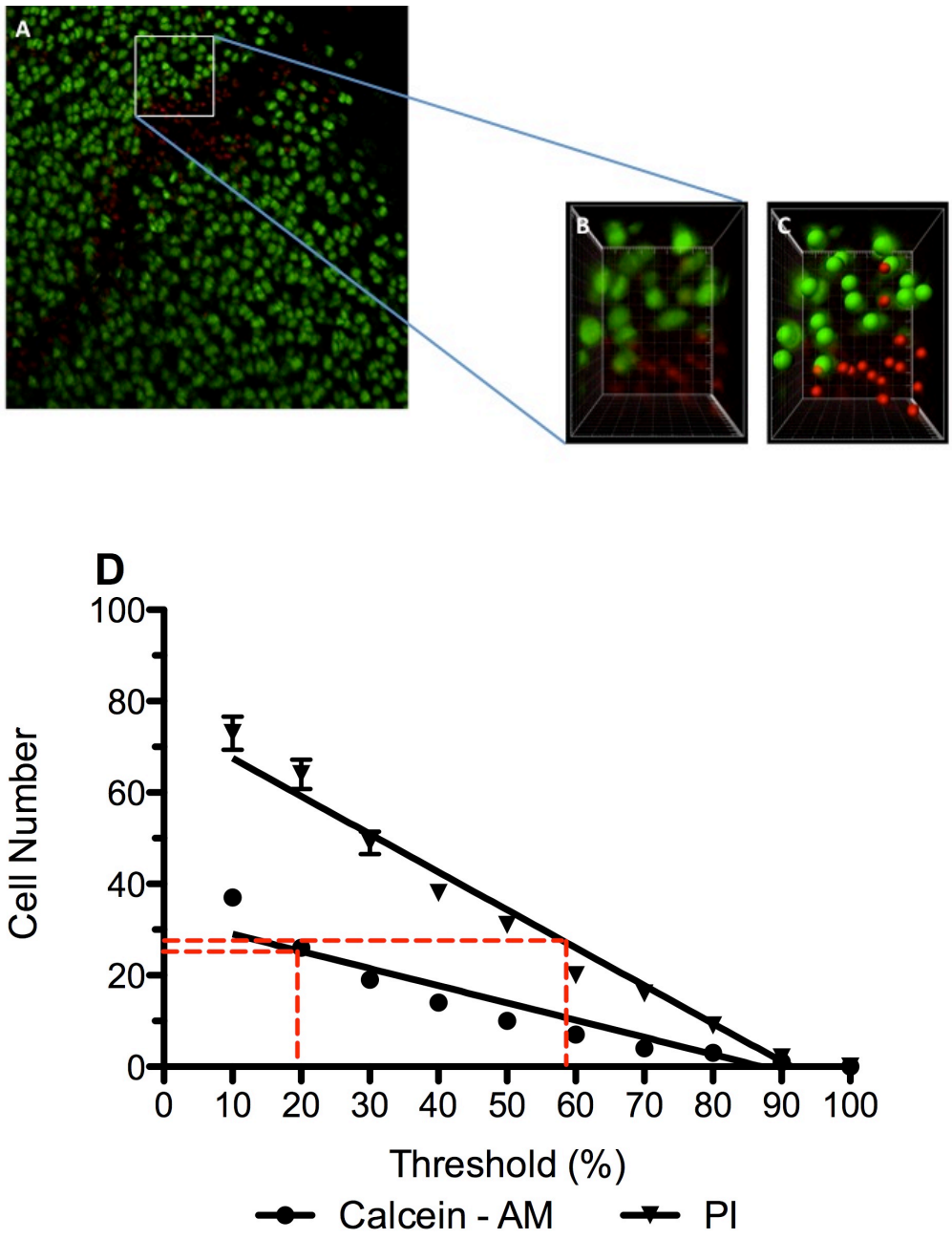


Figure 2.8: Optimisation of spot analysis

A) Bovine explants were stained with 5 μM Calcein-AM and 1 μM Propidium Iodide to visualise viable and non-viable cells respectively. Samples were imaged using CLSM. B) A visually countable number of both viable and non-viable cells was cropped from the CLSM image. C) Spot analysis was applied to the cropped section for both viable and non-viable cells at different threshold percentages. D) Apparent cell number was plotted against threshold percentage and linear regression applied. Actual cell number was used to elucidate the optimal threshold percentage for both calcein-AM and PI.

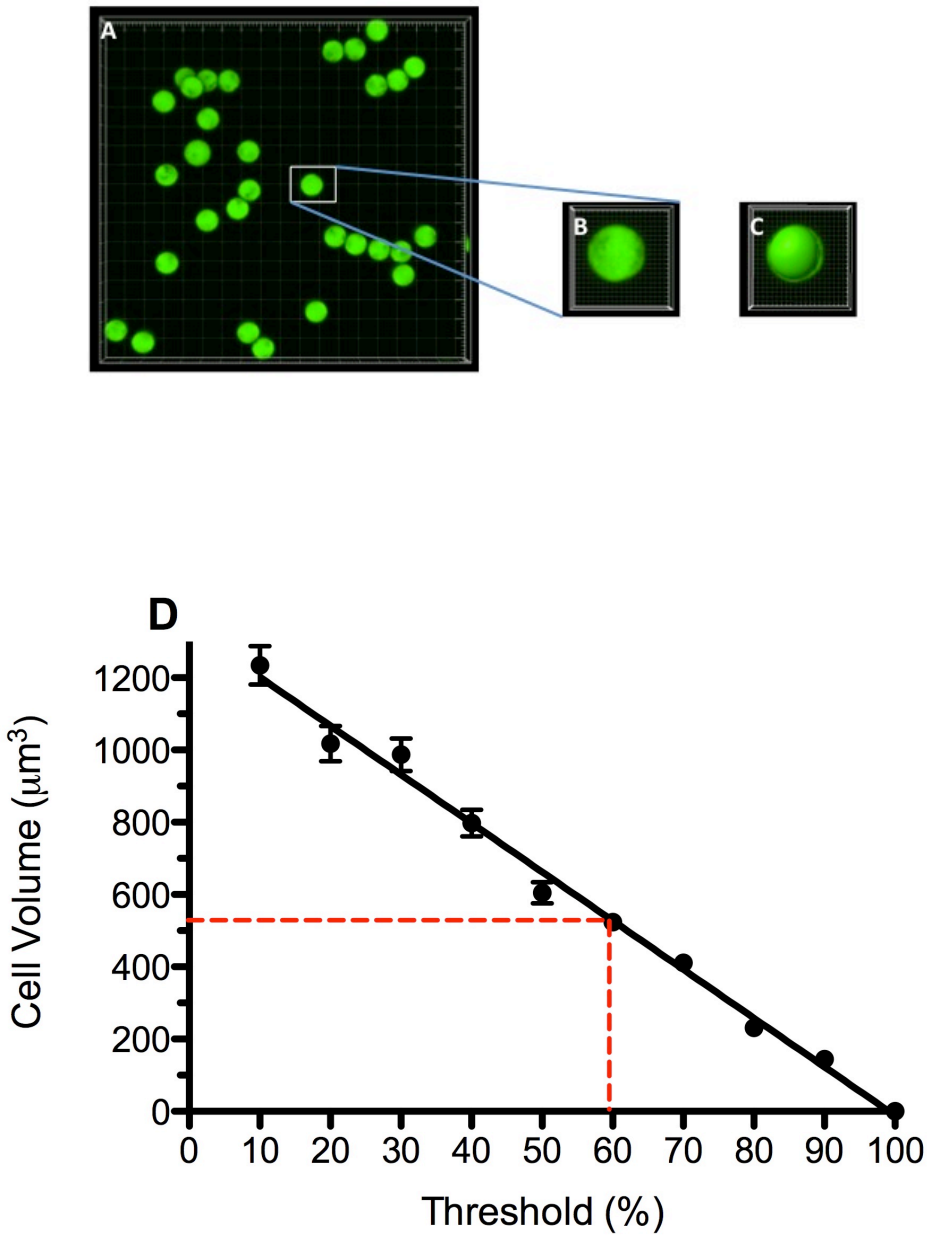


Figure 2.9: Optimisation of volume analysis using beads

A) Latex beads were stained with 5 μM Calcein-AM and imaged using CLSM. B) A single bead was cropped from the CLSM image. C) An isosurface was applied to the cropped bead at different threshold percentages. D) Apparent bead volume was plotted against threshold percentage and linear regression applied. Actual bead volume was used to elucidate the optimal threshold percentage for both calcein-AM and PI.

2.8.3 CLSM Actin Analysis

The organisation of the actin cytoskeleton was studied using ImageJ 1.43U (NIH; USA) with the fluorescence intensity of individual cells determined using Linear Profiling across the cell diameter (*Fig. 2.10*). As the acquisition protocol was standardised between images, thus maximal fluorescence being constant, mean fluorescence intensity ($\bar{I}$) for the population of cells within a sample was obtained in arbitrary fluorescent units. It can be argued that the changing cell volume resultant from osmotic changes (for example when subject to a hyperosmotic challenge) could result in an apparent increase in F-actin, caused by the condensation of the fluorescence (Alvarez-Leefmans, 1995). To combat the possibility of this phenomenon, mean fluorescence intensity was expressed relative to the mean cell volume using the newly termed 'Parker-Qusous Factor' as follows:

F-actin (Fa) is assumed to be proportional to fluorescence ($Fluo$), and similarly fluorescence is assumed to be inversely proportional to cell volume (V) (Alvarez-Leefmans, 1995), thus;

$Fa \propto Fluo$ and $Fluo \propto \frac{1}{V} \rightarrow Fa \propto \frac{Fluo}{V}$

$\bar{I}$ is the fluorescence divided by the radius (r) of the cell, thus;

$$\bar{I} = \frac{Fluo}{r} \rightarrow Fluo = \bar{I}r$$

Volume of a sphere can be calculated as;

$$V = \frac{4}{3}\pi r^3 \rightarrow V \propto r^3$$

Thus;

$$Fa \propto \frac{\bar{I}r}{r^3} \rightarrow Fa \propto \frac{\bar{I}}{r^2}$$

As,

$$V \propto r^3 \rightarrow \sqrt[3]{V} \propto r$$

Thus;

$$Fa \propto \frac{\bar{I}}{\left(\sqrt[3]{V}\right)^2} \rightarrow \frac{\bar{I}}{V^{2/3}}$$

Therefore the Parker-Qusous Factor (PQ) for F-actin is:

$$PQ = \frac{\bar{I}}{V^{2/3}}$$

In this study F-actin was calculated independent of cell volume using the above described Parker-Qusous Factor and expressed in Mauricean Units (MU: arbitrary fluorescent units/μm^3).

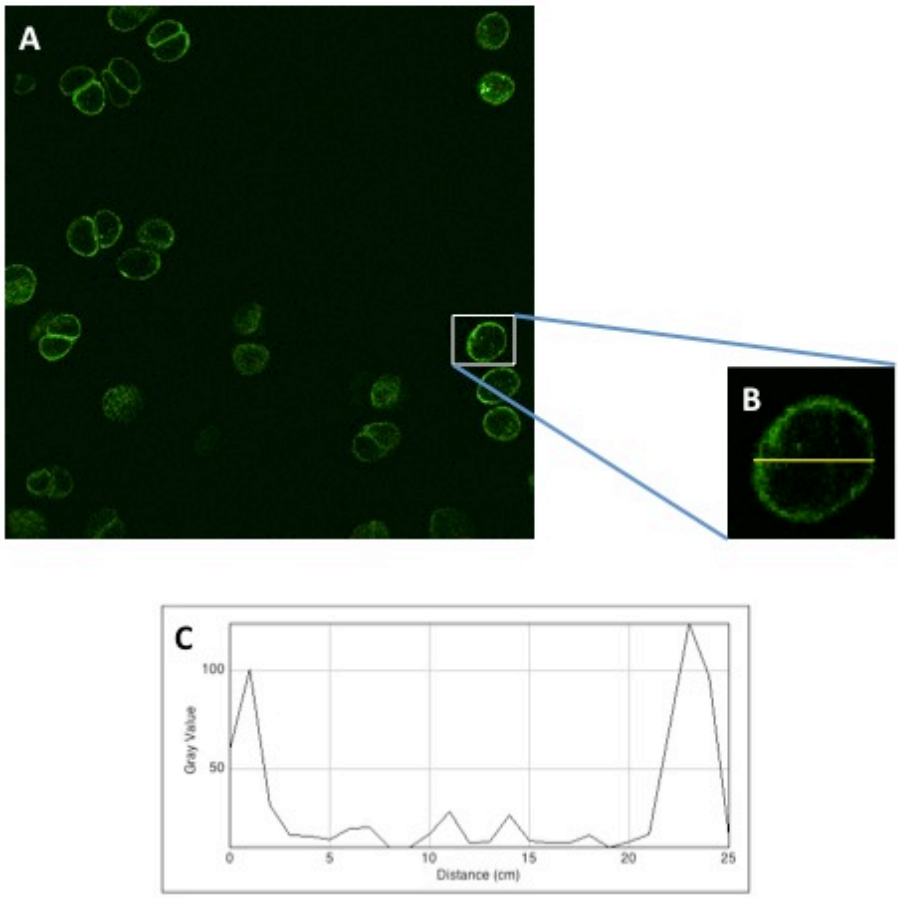


Figure 2.10: Analysis of the organisation of the Actin cytoskeleton by Linear Profiling.

A) Bovine explants were fixed in 4 % paraformaldehyde and stained with alexa fluor 488 phalloidin. Samples were imaged using CLSM. B) Images were imported into ImageJ and analysed by Linear Profiling, whereby lines were drawn horizontally and vertically across the diameter of each individual cell and the fluorescence along the line recorded. C) The fluorescence profile of a single cell.

2.8.4 Mechanical Analysis

Knee impact forces were calculated from the data collected from the force plate (*Section 2.7.2*) using the following equations:

$F_{i-1,i} = F_{i,i+1} - m_i g + m_i a$ (Hutchinson, 1994)

Where;

$F_{i-1,i}$ → force between segments i-1 and I at joint i-1

$F_{i,i+1}$ → force between segments I and i+1 at joint i

m_i → mass of segment i

g → gravity vector

a_i → acceleration vector of C.O.M of segment i

GRF → Ground reaction force

and;

Segment masses (Winter, 1990):

Mass of foot = 1.4 % total mass

Mass of shank = 4.6 % total mass

Mass of thigh = 9.9 % total mass

Therefore;

1) GRF = $Ankle_{Force}$ – ($mass_{foot}$ x g) + ($mass_{foot}$ x speed)

2) $Ankle_{Force}$ = $Knee_{Force}$ – ($mass_{shank}$ x g) + ($mass_{shank}$ x speed)

3) $Knee_{Force}$ = Hip_{Force} – ($mass_{thigh}$ x g) + ($mass_{thigh}$ x speed)

By collecting the GRF from the pressure plate, the mass of the foot (calculated from the published percentage of the total body mass) and the speed, equation 1 can be rearranged to calculate the ankle force. This can then be entered into equation 2 along with shank mass (again calculated from the published percentage of the total body mass) and speed. Equation 2 is then subsequently rearranged to calculate knee force.

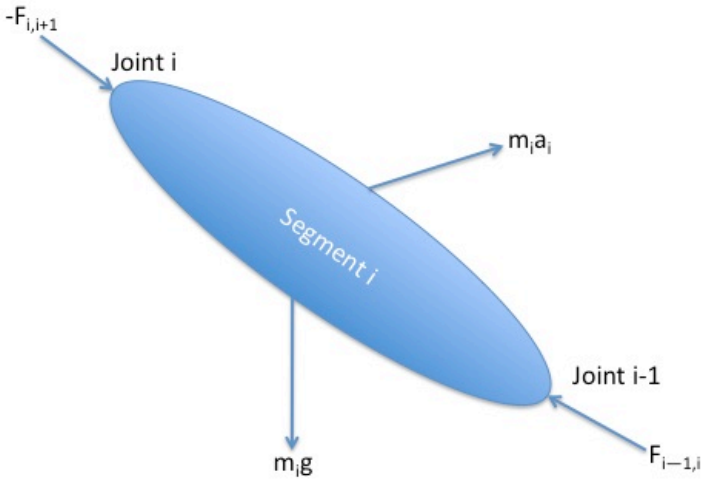


Figure 2.11: Schematic diagram of the forces on a limb segment.

A single limb segment and it's forces, where $F_{i-1,i}$ is the force between segment i and the preceding segment, or in the case of the foot, the force between the ground and the foot, i.e. GRF. $F_{i,i+1}$ is the force between segment i and the succeeding segment. Diagram adapted from (Hutchinson, 1994).

2.9 Statistics

Statistical analysis of Pearson Correlation Coefficient was completed using StatPlus (AnalystSoft; Canada) and all other statistical analysis was performed using Microsoft® Excel 2008 for Mac. The plotting of all graphs and obtaining of graph equations and linear regression statistics (R^2 values given for every equation) were performed using Graphpad Prism 5 (CA, USA).

Linear regression was calculated for viability, volume and actin, to determine rates of cell death, volume decrease and actin polymerisation respectively. Initial (0-4 h) and subsequent (4-48h) rates were calculated (*Fig. 2.12)*.

All values are shown as mean ± standard error of the mean (s.e.m) unless otherwise stated. Two-tailed Student's T-tests were employed to determine the significance of results, which were deemed significantly different if the p value was $p<0.05$ (*), $p<0.01$ (**), or $p<0.005$ (***).

PQ is stated as mean ± error, calculated as follows:

$$\frac{1}{2}\left(\frac{H\bar{I}}{LV^{2/3}}+\frac{L\bar{I}}{HV^{2/3}}\right)$$

Whereby:

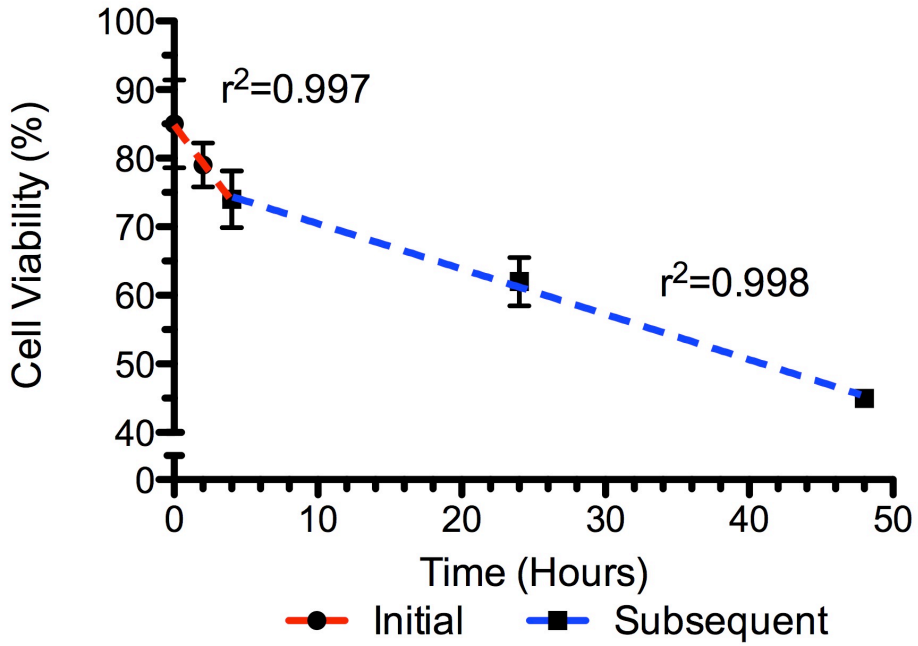
$H\bar{I} \rightarrow$ Mean intensity + s.e.m

$L\bar{I} \rightarrow$ Mean intensity – s.e.m

$HV \rightarrow$ Volume + s.e.m

$LV \rightarrow$ Volume – s.e.m

Experiment repetitions are described in individual legends, where (N) denotes the number of individual joints used and (n) denotes the number of cells, were applicable.



	Rate of chondrocyte death (% hour^{-1})
Initial	2.75±0.14
Subsequent	0.66±0.03

Figure 2.12: Linear regression to determine rate of cell death

Bovine explants were subjected to a single impact from a vertical drop tower and stained with 5 μM Calcein-AM and 1 μM Propidium Iodide to visualise viable and non-viable cells respectively. Samples were imaged using CLSM prior to and at 2, 4, 24 and 48 h post impact. Spot analysis was applied for both viable and non-viable cells and percentage cell viability plotted against time. Linear regression was applied for both initial (0-4 h) and subsequent (4-48 h) cell death.

Chapter 3: Osmotic Sensitivity of *In Situ* Chondrocytes in Response to Mechanical Trauma

3.1 Introduction

Mechanical loading is known to be essential for the maintenance of healthy articular cartilage tissue, whereby production of an extracellular matrix (ECM) of sufficient durability to withstand the loading is induced (Helminen, 1987). However, excessive loading, from mechanical trauma or repetitive high impact exercise, is observed to induce cartilage damage and chondrocyte apoptosis in addition to increasing the risk of osteoarthritis development (OA; (Oliveria *et al*., 1999, Tew *et al*., 2000). Excessive loading is noted to induce tissue swelling, indicative of damage to the collagen network (Jeffrey *et al*., 1995), which is thought to play a role in the loss of viable chondrocytes within OA tissue, whereby over-hydrated cells are thought to be more sensitive to mechanical stresses (Bush *et al*., 2005). Additionally, cytoskeletal actin filaments (F-actin), known to provide mechanical cellular support and play a role in maintaining chondrocyte durability and strength, are reported to be disassembled in response to mechanical trauma (Blain, 2009), such that there is a loss of F-actin fibres and reorganisation of the filament distribution from a cortical to a more scattered arrangement (Knight *et al*., 2006, Parkkinen *et al*., 1995). It is further reported that mechanical trauma induces release of cytokines, including pro-inflammatory cytokines IL-1β and MCP-1, and anti-inflammatory cytokine IL-10. IL-1β is known to disrupt F-actin and bring about ECM degradation by inducing a decrease in proteoglycan (PG) synthesis and an increase in catabolic enzyme release (Liu *et al*., 1994, Rowan and Young, 2007, Westacott and Sharif, 1996).

Joint articulation, as a consequence of daily activity compresses cartilage tissue, inducing the expulsion of water and thus an increase in tissue osmolarity. It is therefore postulated that tissue *in vivo* is at a higher osmotic pressure at the end of the diurnal cycle. Whilst it has been observed that decreased extracellular osmolarity and thus cell swelling, such as observed in early-stage OA, results in chondrocyte deformation, and loss of viability when the tissue is subjected to mechanical trauma (Bush and Hall, 2001b), it is unknown what effect increased extracellular osmolarity, such as

resultant from daily joint articulation, will have on cartilage integrity when subjected to similar abnormal loading.

3.1.1 Chapter Aims

This study aims to build on the previous work described, investigating the effects of prolonged static loading, as simulated by hyperosmotic challenge, on *in vitro* cartilage integrity and inflammation post mechanical impact.
Specifically by:

1. Investigation of changes in chondrocyte viability and volume in response to single mechanical impact.
2. Investigation of the actin cytoskeletal response to mechanical impact.
3. Determining the changes in inflammatory cytokine concentrations in articular cartilage post mechanical impact.
4. Investigation of the role of extracellular osmolarity in responses observed post mechanical impact.

3.2 Results

3.2.1 The Osmotic Sensitivity of Articular Chondrocytes

The effects of increased extracellular osmolarity on the integrity of articular chondrocytes post mechanical impact was investigated initially by determining cellular responses to an osmotic gradient. Thus, articular cartilage explants were dissected from bovine metacarpal/metatarsal phalangeal joints and incubated at 37°C for 1 h with DMEM of 280, 320, 360 or 380 mOsm. Subsequently, samples were subjected to a single mechanical impact as previously described (*See Materials and Methods*) and analysed by CLSM for viability and volume.

Mechanical impact induced chondrocyte death under control, isotonic conditions, whereby a biphasic decrease in cell viability was observed (*Fig. 3.1*). This observation was characterised by rapid initial cell death (2.75±0.14 $\%.h^{-1}$) followed by slower subsequent cell death (0.66±0.03 $\%.h^{-1}$). Increasing extracellular osmolarity to 320 and 360 mOsm displayed a chondroprotective effect, reducing the rate of both initial (2.00±0.29 $\%.h^{-1}$, 1.25±0.23 $\%.h^{-1}$) and subsequent cell death (0.23±0.01 $\%.h^{-1}$, 0.13±0.00 $\%.h^{-1}$), but biphasic cell death was still observed. In contrast, increasing extracellular osmolarity to 380 mOsm resulted in no significant ($p>0.05$) cell death over 48 h post mechanical impact (viability of 90±2.12 % at 0 h to 90±2.20 % at 48 h), abolishing both the initial (0.00±0.03 $\%.h^{-1}$) and the subsequent (0.00±0.06 $\%.h^{-1}$) phases of chondrocyte death (*Fig. 3.2*).

Chondrocyte volume was noted to be 716.14±37.81 μm^3 within *in situ* cartilage under isotonic conditions. Following mechanical impact, a biphasic decrease in cell volume was observed, with a rapid initial rate of 34.38±1.34 $\mu m^3.h^{-1}$ and a slower subsequent rate of 2.87±0.85 $\mu m^3.h^{-1}$. This observation was termed Impact Induced Volume Decrease (IIVD). Increasing extracellular osmolarity to 320, 360 and 380 mOsm unsurprisingly decreased cell volume prior to mechanical impact (*Fig. 3.3*), to 645.12±46.10 μm^3, 587.64±23.22 μm^3 and 561.46±25.49 μm^3 respectively. However, whilst this initial decrease in chondrocyte volume

prior to mechanical impact significantly ($p<0.005$) decreased the initial rate of IIVD (320 mOsm: 28.00±0.38 $\mu m^3.h^{-1}$, 360 mOsm: 28.00±0.79 $\mu m^3.h^{-1}$, 380 mOsm: 26.95±1.12 $\mu m^3.h^{-1}$), IIVD was still observed (*Fig. 3.3*). Additionally, increased extracellular osmolarity significantly ($p<0.005$) decreased the subsequent rate of IIVD (320 mOsm: 2.14±0.18 $\mu m^3.h^{-1}$, 360 mOsm: 0.97±0.06 $\mu m^3.h^{-1}$, 380 mOsm: 00.54±0.05 $\mu m^3.h^{-1}$), where 380 mOsm was noted to induce a near elimination of the subsequent phase (*Fig. 3.3*). These data thus demonstrated that 380 mOsm DMEM had the most effective chondroprotective effects and was used hereafter to model increased extracellular osmolarity.

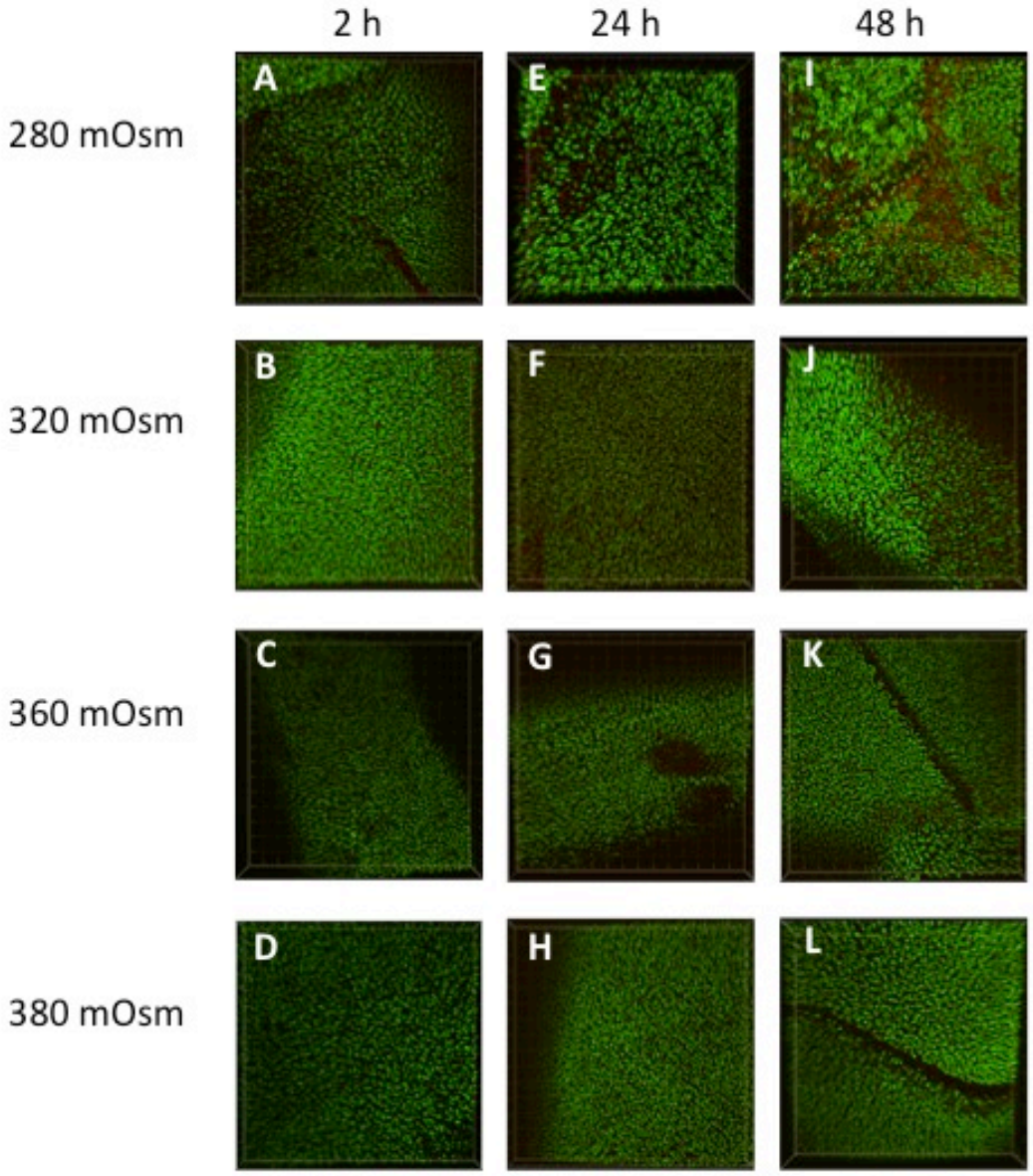
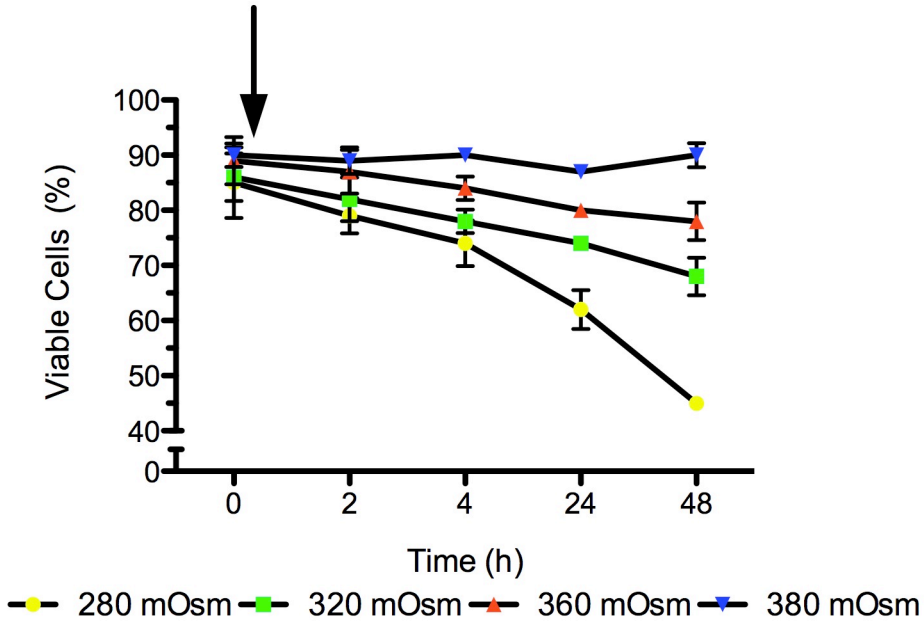


Figure 3.1: The role of osmolarity on chondrocyte viability following mechanical impact.

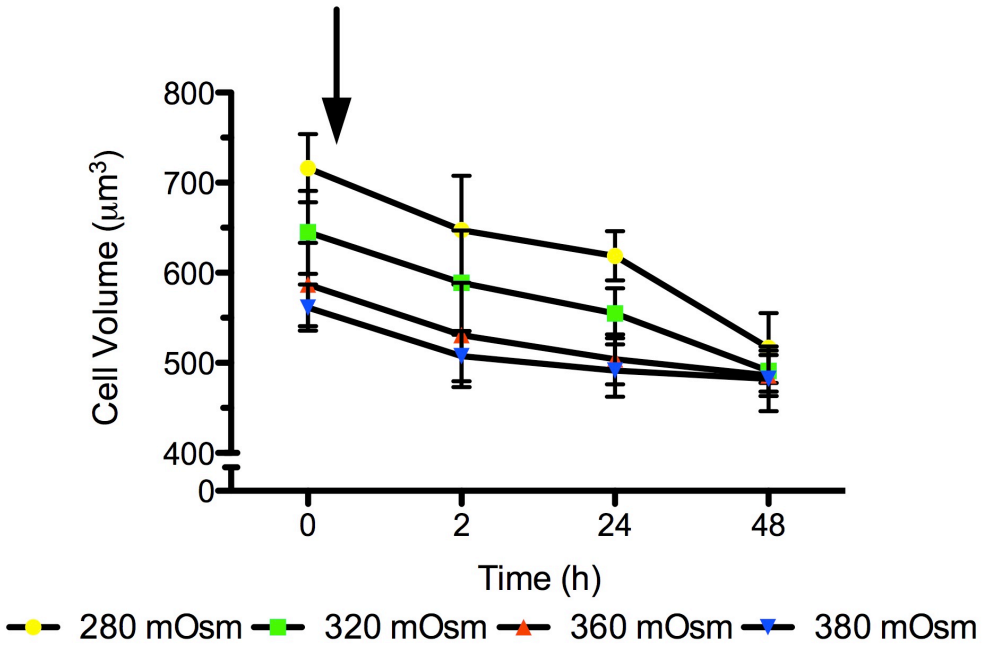
Cartilage explants were pre-treated for 1 h with control isotonic (A,E,I) or hyperosmotic DMEM of 320 (B,F,J), 360 (C,G,K) or 380 (D,H,L) mOsm and subjected to a single mechanical impact. Samples were loaded with Calcein AM (5 μM) and PI (1 μM) and visualised using CLSM at 2 h (A-D), 24 h (E-H) and 48 h (I-L) post mechanical impact. Imaris Spot analysis was used to quantify the number of viable (green) and non-viable (red) cells.



Osmolarity (mOsm)	Initial rate of chondrocyte death (%.h^{-1})	Subsequent rate of chondrocyte death (%.h^{-1})
280	2.75±0.14	0.66±0.03
320	2.00±0.29 *	0.23±0.01 ***
360	1.25±0.23 ***	0.13±0.00 ***
380	0.00±0.03 ***	0.00±0.06 ***

Figure 3.2: Hyperosmotic pre-treatment is chondroprotective post-mechanical impact.

*Cartilage explants were pre-treated for 1 h with control isotonic or hyperosmotic DMEM of 320, 360 or 380 mOsm and subjected to a single mechanical impact. Samples were loaded with Calcein AM (5 μM) and PI (1 μM) and visualised using CLSM prior to and at 2 h, 24 h and 48 h post mechanical impact. Imaris Spot analysis was used to quantify the number of viable and non-viable cells. Biphasic cell death was observed under all osmotic conditions except 380 mOsm. N=12 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005 all vs. control (280 mOsm). Arrow denotes impact point.*



Osmolarity (mOsm)	Initial rate of chondrocyte volume decrease ($\mu m^3.h^{-1}$)	Subsequent rate of chondrocyte volume decrease ($\mu m^3.h^{-1}$)
280	34.38±1.34	2.87±0.85
320	28.00±0.38 ***	2.14±0.18 ***
360	28.00±0.79 ***	0.97±0.06 ***
380	26.95±1.12 ***	0.54±0.05 ***

Figure 3.3: Hyperosmotic pre-treatment decreases chondrocyte volume post-mechanical impact.

*Cartilage explants were pre-treated for 1 h with control isotonic or hyperosmotic DMEM of 320, 360 or 380 mOsm and subjected to a single mechanical impact. Samples were loaded with Calcein AM (5 μM) and visualised using CLSM prior to and at 2 h, 24 h and 48 h post mechanical impact. Isosurface volume analysis was used to quantify cell volume. Hyperosmotic challenge decreased cell volume prior to impact. A biphasic decrease in cell volume was observed under all osmotic conditions. N=12 and n=60 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005 all vs. control (280 mOsm). Arrow denotes impact point.*

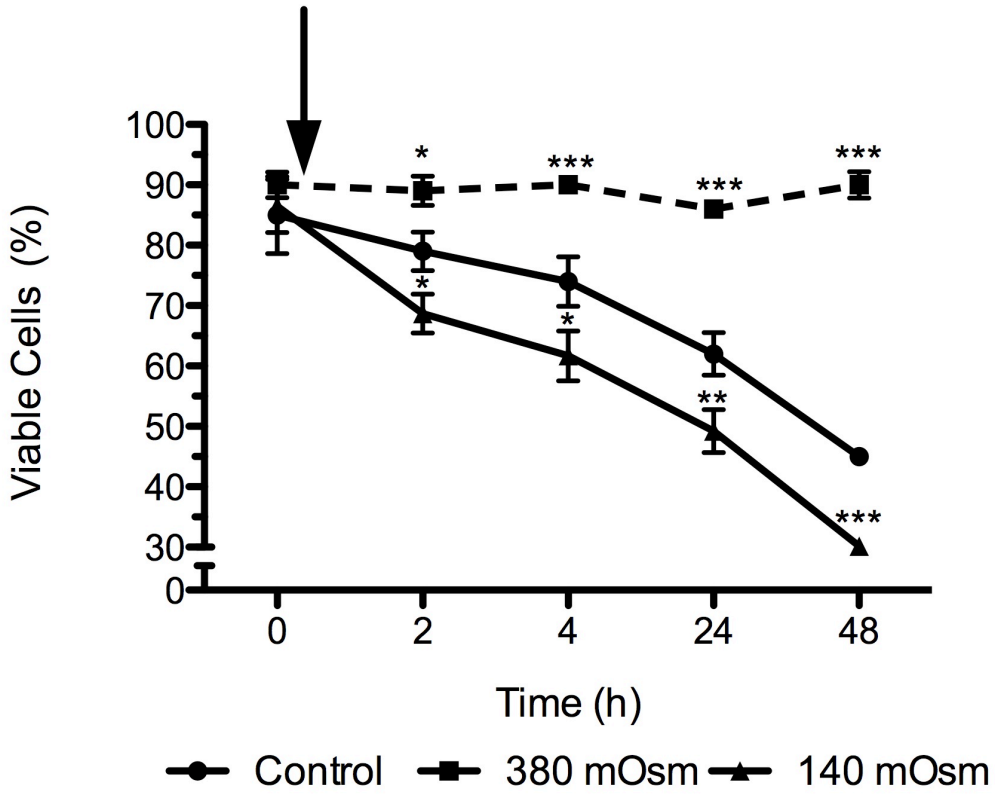
3.2.2 Extracellular Osmolarity and Mechanical Impact

Previously 380 mOsm was determined to be the most effective osmolarity for chondroprotection, this osmolarity was thus used for the purposes of this study as a hyperosmotic model of 'evening' articular cartilage that has been subjected to daily joint articulation. Additionally, OA tissue is known to be over hydrated (Bush and Hall, 2003), thus a hypo-osmotic challenge of 140 mOsm was employed to induce cell swelling and thus act as a simulation for OA tissue. These simulations were further investigated and compared to determine the consequences of changes in the extracellular osmotic environment on articular cartilage responses to mechanical loading. Consequently, samples were pre-treated with hypo-osmotic (140 mOsm), isotonic (280 mOsm) or hyperosmotic (380 mOsm) DMEM prior to mechanical impact and samples analysed for cell viability and volume by CLSM.

It was observed that hypo-osmotic and hyperosmotic challenge had opposing effects on cell viability post mechanical impact, whereby hyperosmotic challenge exhibited chondroprotective actions (90.00±2.20 % viability at 48 h), and hypo-osmotic challenge resulted in an increase in cell death (30.16±0.99 % viability at 48 h; Control: 45.00±1.21 %). Furthermore, whilst hyperosmotic challenge abolished the biphasic cell death observed in control samples, hypo-osmotic challenge increased both the initial rate (6.21±1.56 $\%.h^{-1}$) and the subsequent rate (0.78±0.05 $\%.h^{-1}$) of cell death induced by mechanical impact (*Fig. 3.4*).

As expected, hypo-osmotic challenge was seen to significantly increase cell volume ($p<0.005$) prior to mechanical impact (*Fig. 3.5*) to 923.27±37.81 μm^3 when compared to control samples (716.14±35.82 μm^3; *Fig. 3.6A*). Conversely hyperosmotic treatment significantly decreased cell volume ($p<0.01$) to 578.46±25.49 μm^3 prior to mechanical impact (*Fig. 3.5 & 3.6*). The biphasic IIVD observed under control conditions was evident in both hypo-osmotic and hyperosmotic conditions, however both the initial rate (54.50±0.04 $\mu m^3.h^{-1}$) and the subsequent rate (4.05±0.49 $\mu m^3.h^{-1}$) of IIVD were observed to be significantly faster in hypo-osmotic conditions (*Fig.*

3.6). Hyperosmotic challenge had the opposing action, decreasing the initial rate of IIVD, and severely reducing the subsequent rate, whereby despite the lower cell volume prior to impact, the volume of control (516.52±38.86 μm^3) and hyperosmotic samples (482.49±35.93 μm^3) were not significantly different ($p>0.05$) at 48 h post impact (*Fig. 3.6*).



Osmolarity (mOsm)	Initial rate of chondrocyte death (%.h^{-1})	Subsequent rate of chondrocyte death (%.h^{-1})
280	2.75±0.14	0.66±0.03
380	0.00±0.03 ***	0.00±0.06 ***
140	6.21±1.56 **	0.78±0.05 ***

Figure 3.4: Hyperosmotic challenge protects against cell death post mechanical impact.

*Cartilage explants were pre-treated for 1 h with control isotonic, hypo-osmotic (140 mOsm) or hyperosmotic (380 mOsm) DMEM and subjected to a single mechanical impact. Samples were loaded with Calcein AM (5 μM) and PI (1 μM) and visualised using CLSM prior to and at 2 h, 24 h and 48 h post mechanical impact. Imaris spot analysis was used to quantify cell viability. Hyperosmotic treatment was seen to protect against cell death. N=12 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005 all vs. control. Arrow denotes impact point.*

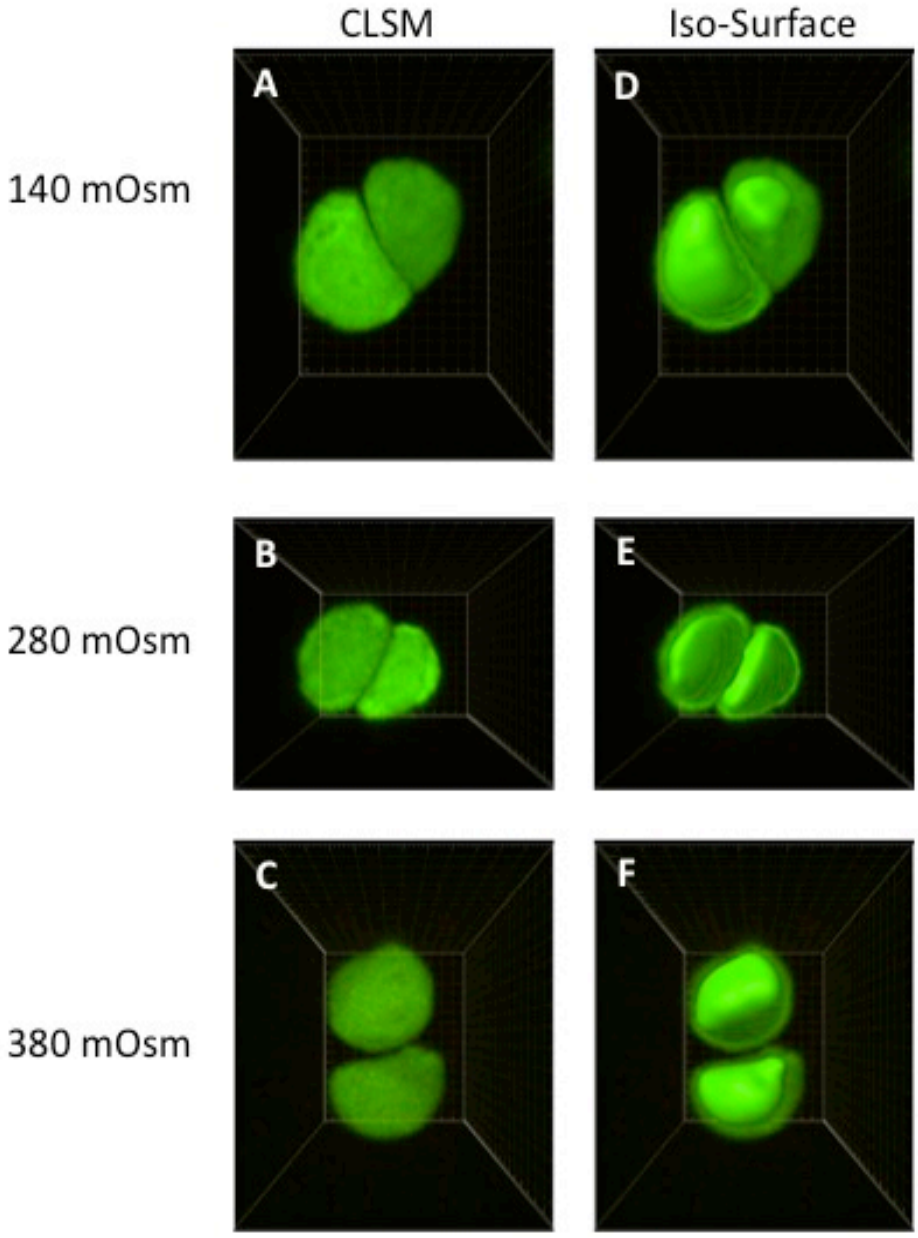
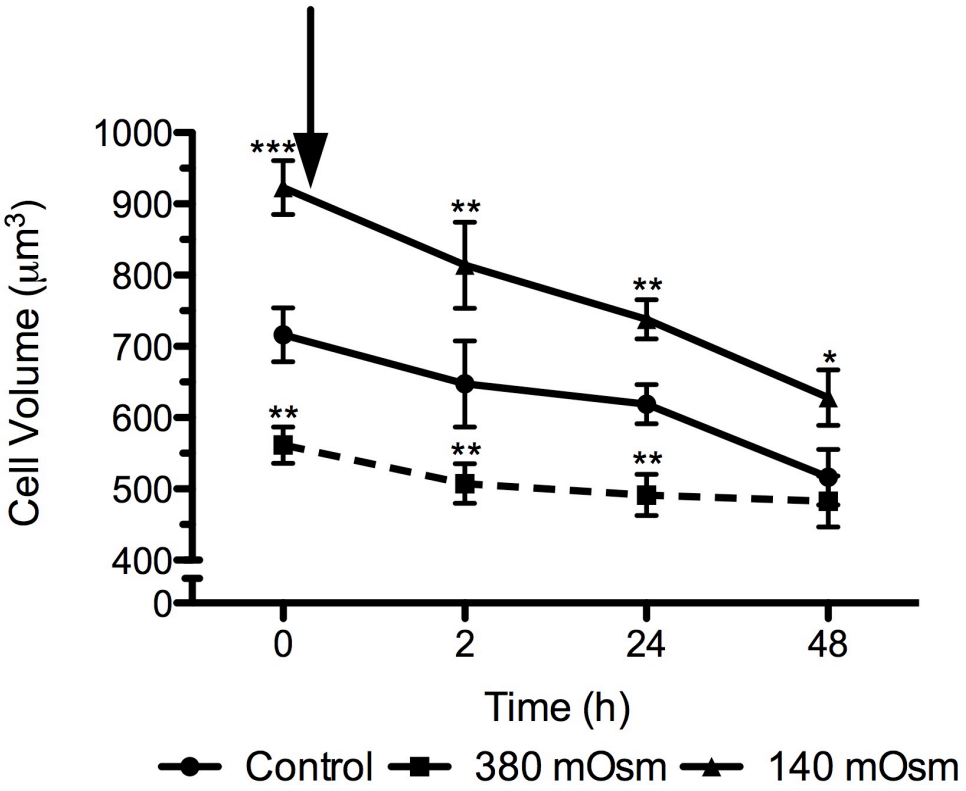


Figure 3.5: Isosurface volume analysis of cells.

Cartilage explants were treated for 1 h with control isotonic (B & E), hypo-osmotic (140 mOsm; A & D) or hyperosmotic (380 mOsm; C & F). Samples were stained with Calcein AM (5 μM) and visualised using CLSM. Isosurface volume analysis was used to determine cell volume.



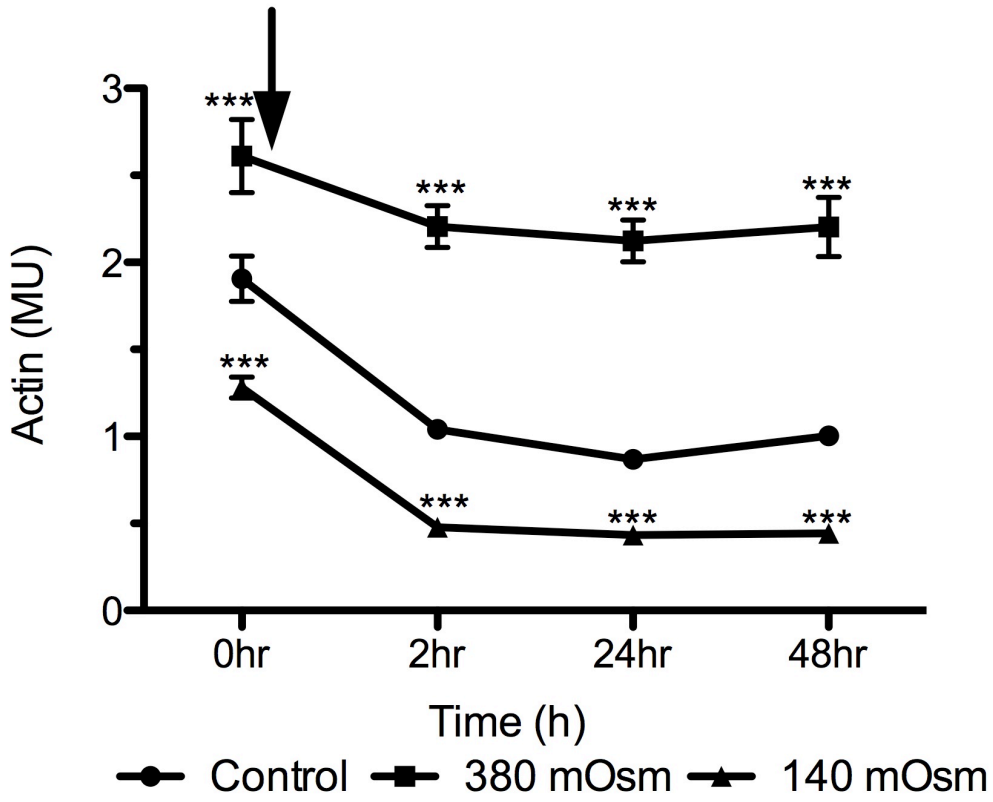
Osmolarity (mOsm)	Initial rate of chondrocyte volume decrease ($\mu m^3.h^{-1}$)	Subsequent rate of chondrocyte volume decrease ($\mu m^3.h^{-1}$)
280	34.38±0.03	2.87±0.85
380	26.95±1.12 ***	0.54±0.05 ***
140	54.50±0.04 ***	4.05±0.49 ***

Figure 3.6: Chondrocyte volume decreases biphasically post-mechanical impact. *Cartilage explants were pre-treated for 1 h with control isotonic, hypo-osmotic (140 mOsm) or hyperosmotic (380 mOsm) challenges and subjected to a single mechanical insult. Samples were loaded with Calcein AM (5 μM) and visualised using CLSM prior to and at 2 h, 24 h and 48 h post mechanical impact. Isosurface volume analysis was used to quantify cell volume. A biphasic decrease in cell volume was observed under all osmotic conditions. Initial decrease was significantly faster for hypo-osmotic treatment. N=12 and n=60 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005. Arrow denotes impact point.*

3.2.3 The Responses of Filamentous Actin to Mechanical Impact

The F-actin cytoskeleton is a dynamic structure known to be involved in both mechanotransduction, whereby mechanical stimuli are converted into a biochemical response, and in maintaining structural cell integrity (Blain, 2009, Guilak *et al*., 1995, Wang *et al*., 1993). Furthermore, F-actin organisation is noted to be less defined in OA tissue, thus possibly contributing to the defective function found in OA (Blain, 2009). The responses of F-actin to mechanical impact were investigated, post hypo-osmotic and hyperosmotic challenge to clarify the consequences of altered extracellular osmolarity, either due to OA or daily joint articulation, on F-actin stability post mechanical impact. Articular cartilage samples were fixed with 4% paraformaldehyde and stained for F-actin with Alexa Fluor 488 Phalloidin (5 μl/ml) prior to and 2, 24 and 48 h post mechanical impact. F-actin was imaged using CLSM and analysed using linear profiling and the PQ Factor, as previously described (*See Methods and Materials*).

Prior to mechanical impact, hypo-osmotic challenge induced in an initial decrease in F-actin to 1.08±0.06 MU, when compared to control values of 1.91±0.13 MU, whereas hyperosmotic treatment was observed to induce an increase in F-actin prior to 2.61±0.21 MU (*Fig. 3.7*). Mechanical impact was noted to induce a rapid decrease in F-actin (0.43±0.03 $MU.h^{-1}$) under control conditions (*Fig. 3.7*), termed Impact Induced Actin Decrease (IIAD), between 0 and 2 h post impact. No significant ($p>0.05$) alterations in F-actin were observed between 2 – 48 h post impact. Additionally, whilst IIAD was not observed to be significantly affected by hypo-osmotic challenge (0.40-±0.03 $MU.h^{-1}$), hyperosmotic challenge was noted to reduce the rate of IIAD by more than 50 % to 0.20±0.04 $MU.h^{-1}$ (*Fig. 3.7*).



Osmolarity (mOsm)	Initial rate of actin polymerisation ($MU.h^{-1}$)	Subsequent rate of actin polymerisation ($MU.h^{-1}$)
280	-0.43 ± 0.03	0.00 ± 0.00
380	-0.20 ± 0.04 ***	0.01 ± 0.00
140	-0.40 ± 0.03 *	-0.01 ± 0.00

Figure 3.7: F-actin decreases post-mechanical impact.

*Cartilage explants were pre-treated for 1 h with control isotonic, hypo-osmotic (140 mOsm) or hyperosmotic (380 mOsm) challenges and subjected to a single mechanical impact. Samples were fixed with 4 % paraformaldehyde and stained with Alexa Fluor 488 phalloidin (5 μl/ml). Cells were visualised using CLSM prior to and at 2 h, 24 h and 48 h post mechanical impact. Linear profiling analysis was used to quantify f-actin, expressed relative to volume changes. An initial decrease in actin polymerisation was observed under all osmotic conditions, a significantly less acute decrease was seen under hyperosmotic conditions. N=12 and n=60 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005 all vs. control. Arrow denotes impact point.*

3.2.4 The Cytokine Response to Mechanical Impact

Various cytokines are known to have a role within the OA pathology, whereby they induce the breakdown of the extracellular matrix (ECM) via increased production of matrix degrading enzymes such as matrix metalloproteinases (MMPs) (Rowan and Young, 2007, Westacott and Sharif, 1996). Given this sensitivity of articular cartilage to inflammatory cytokines, the effect of mechanical impact on cytokine release was investigated.

Supernatant samples were collected immediately and at 2, 24 and 48 h post mechanical impact and analysed by ELISA for cytokine concentration, whereby cytokine concentrations were detected as pg/ml and subsequently adjusted for weight. Articular cartilage was observed to release IL-1β (33.53±4.20 pg/ml/g), IL-10 (80.98±24.30 pg/ml/g) and MCP-1 (50.02±6.4 pg/ml/g) prior to impact (*Fig. 3.8*).

Mechanical impact induced an increase in cytokines, whereby pro-inflammatory cytokine IL-1β exhibited a near 6 fold increase and MCP-1 a 0.5 fold increase immediately post impact (*Fig. 3.8A*), whilst with the anti-inflammatory cytokine IL-10 a near 6 fold increase at 24 h post impact was observed (*Fig. 3.8B*). When relative to baseline non-impact levels, mechanical impact was noted to induce an increase in pro-inflammatory cytokines IL-1β and MCP-1 immediately post impact, followed by a decline back to baseline levels by 24 h (*Fig. 3.9A&C*). The anti-inflammatory cytokine IL-10 was not significantly ($p>0.05$) increased immediately, but was increased 3 fold by 2 h before peaking at 24 h post impact and starting to decline, although still elevated nearly 5 fold at 48 h (*Fig. 3.9B*).

Whilst neither hyperosmotic or hypo-osmotic challenges were observed to effect the profile of cytokine release induced by mechanical impact, whereby pro-inflammatory cytokines IL-1β and MCP-1 peaked immediately post impact at 0 h before declining to baseline and IL-10 peaked at 24 h, hyperosmotic challenge notably decreased the magnitude of cytokine production, decreasing maximal IL-1β release at 0 h by 60 %, maximal

MCP-1 at 0 h by 10 % and maximal IL-10 at 24 h by 19 % (*Fig. 3.9*). Conversely, hypo-osmotic challenge increased maximal cytokine production, increasing IL-1β at 0 h by 31 %, MCP-1 at 0 h by 98 % and IL-10 at 24 h by 33 % (*Fig. 3.9*).

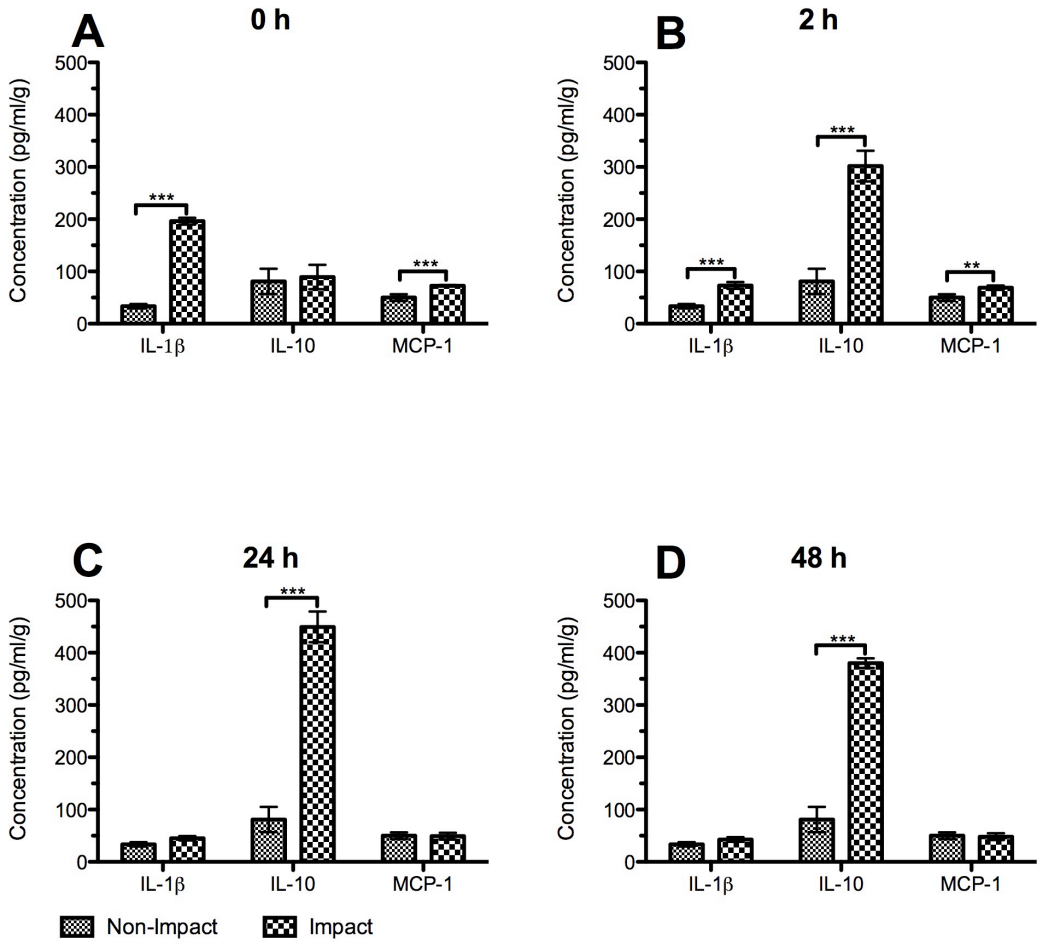


Figure 3.8: Inflammatory cytokines increase post-mechanical impact.

*Cartilage explants were subjected to a single mechanical impact. Supernatant samples immediately following (A) and at 2 h (B), 24 h (C) and 48 h (D) post mechanical impact were analysed by ELISA. Mechanical impact was observed to increase inflammatory cytokines. IL-1β and MCP-1 were increased for up to 2 h post impact, IL-10 was increased from 2 – 48 h, peaking at 24 h. N=12 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005.*

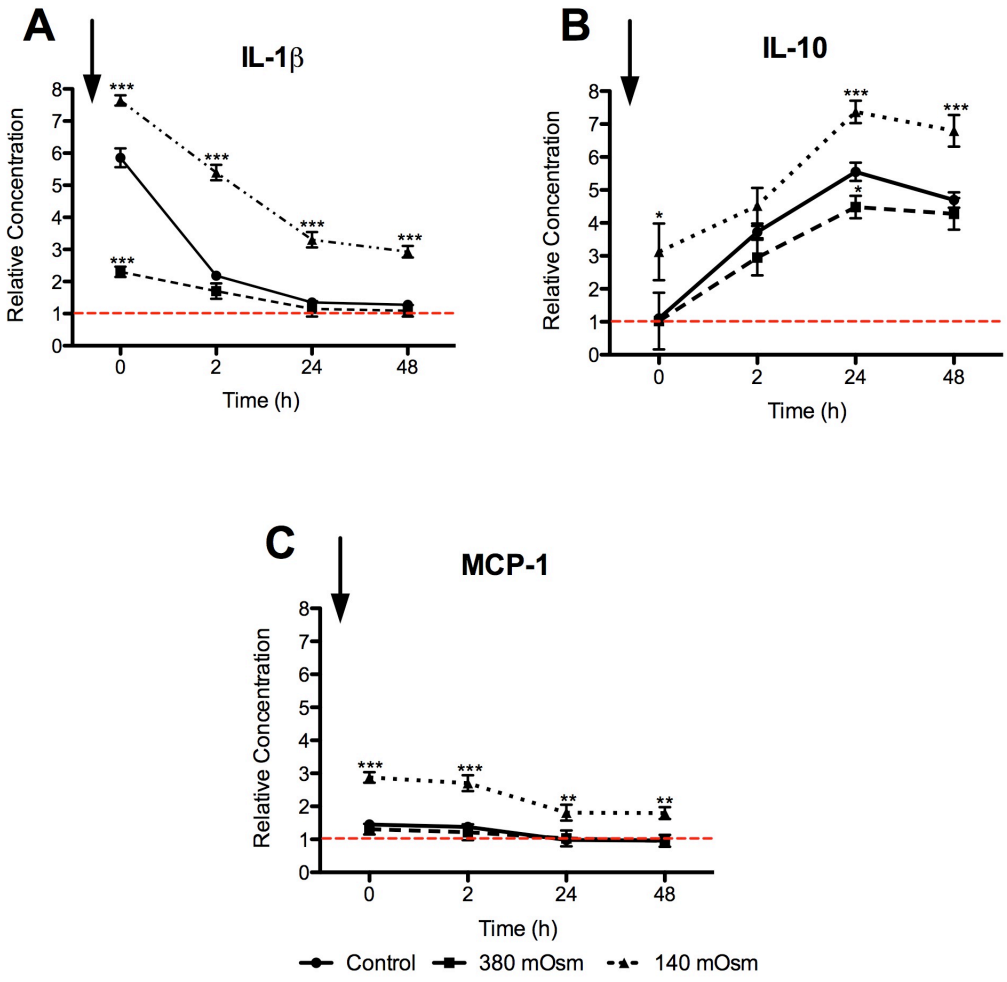


Figure 3.9: Hypo-osmotic conditions increase inflammatory cytokines post-mechanical impact.

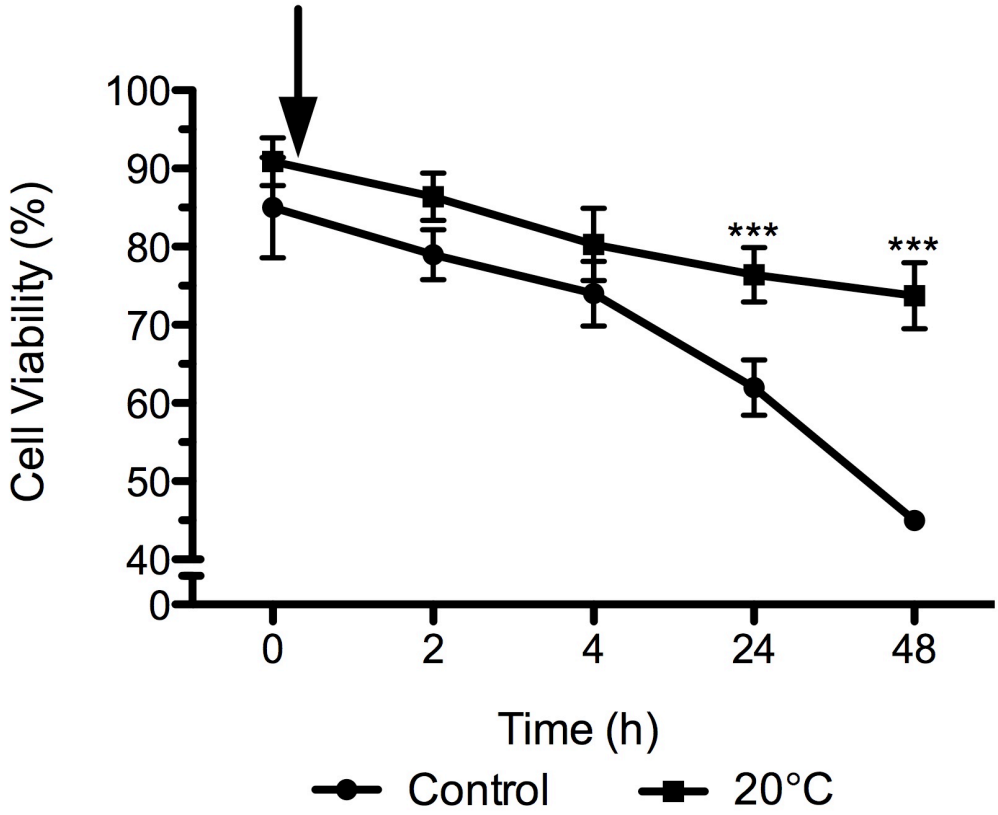
*Cartilage explants were pre-treated for 1 h with control isotonic, hypo-osmotic (140 mOsm) or hyperosmotic (380 mOsm) challenges and subjected to a single mechanical impact. Supernatant samples immediately following and at 2 h, 24 h and 48 h post mechanical impact were analysed by ELISA and cytokine concentration expressed relative to non-impacted control samples. Hypo-osmotic treatment significantly increased IL-1β (A), IL-10 (B) and MCP-1 (C) over the whole time course. Hyperosmotic significantly decreased peak IL-1β and peak IL-10. N=12 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005 all vs. control. Arrow denotes impact point.*

3.2.5 Chondrocyte Death and IIVD as "Active" Processes

Cell death traditionally is thought to occur by two mechanisms, passive uncontrolled necrosis or programmed, active apoptosis. Mechanical impact induced biphasic cell death is yet to be definitively described as either apoptosis or necrosis, or a combination. To investigate the role of active cell death post mechanical impact, articular cartilage explants were incubated at a lowered temperature of 20°C or a control of 37°C prior to and post mechanical impact and subsequently analysed by CLSM for viability prior to and at 2, 4, 24 and 48 h post mechanical impact. Additionally, as the mechanism of IIVD is yet unknown, to determine whether the process is a passive cell response or an active cellular process, samples were also analysed for volume post mechanical impact.

Decreased temperature was observed to have no effect on the rate of initial cell death, whereby cell viability was not significantly ($p>0.05$) different at 4 h post impact (80.29±4.61 %) to control 37°C (74.01±4.12 %). However, decreased temperature was observed to significantly ($p<0.005$) decrease the subsequent rate (0.15±0.06 $\%.h^{-1}$) of cell death compared to control temperature (0.66±0.03 $\%.h^{-1}$), resulting in an increased cell viability at 48 h of 73.73±4.24 % compared to 45.01±0.99 % (*Fig. 3.10*).

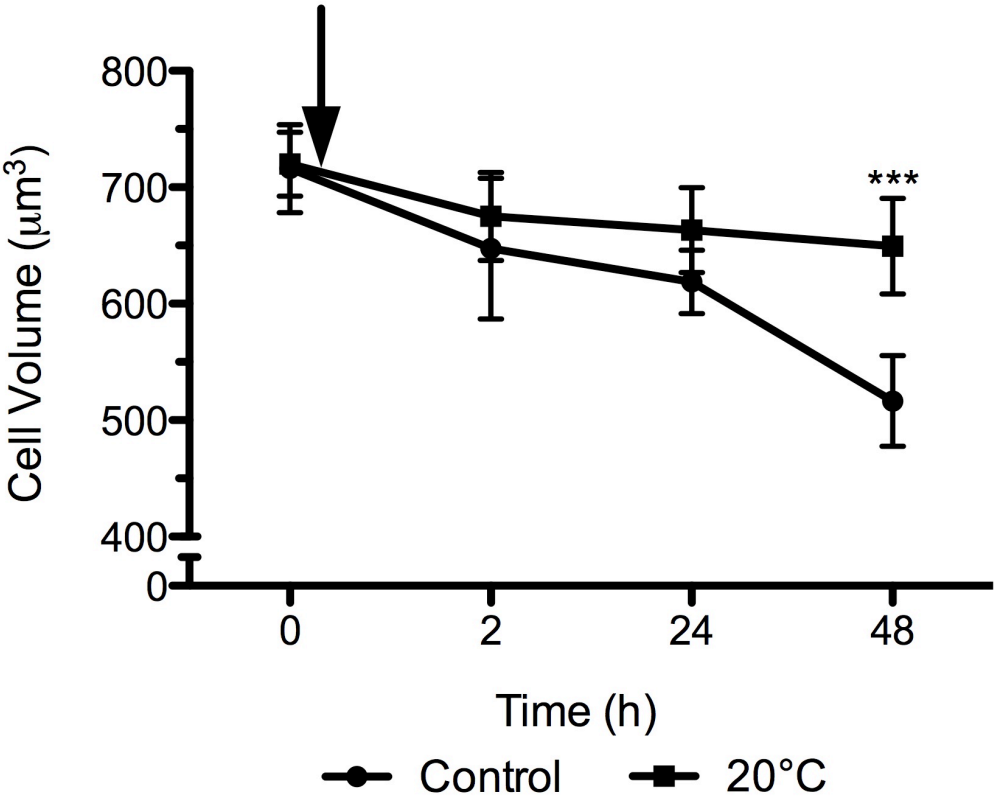
In addition, 20°C incubation was noted to significantly ($p<0.01$) decrease the initial rate of IIVD, to 22.50±0.23 $\mu m^3.h^{-1}$, and the subsequent rate to 0.56±0.01 $\mu m^3.h^{-1}$, thus implying an active component to both phases of IIVD (*Fig. 3.11*).



Temperature (°C)	Initial rate of chondrocyte death (%.h^{-1})	Subsequent rate of chondrocyte death (%.h^{-1})
37 (control)	2.75 ± 0.14	0.66 ± 0.03
20	2.65 ± 0.24	0.15 ± 0.06 ***

Figure 3.10: Cell death post impact is temperature dependent.

*Cartilage explants were kept at control 37°C or 20°C and subjected to a single mechanical impact. Samples were loaded with Calcein AM (5 μM) and PI (1 μM) and visualised using CLSM prior to and at 2 h, 24 h and 48 h post mechanical impact. Imaris spot analysis was used to quantify cell viability. Decreased temperature was observed to protect against cell death, with a significantly slower rate of subsequent cell death. N=12 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005 all vs. control.* Arrow denotes impact point.



Temperature (°C)	Initial rate of chondrocyte volume decrease ($\mu m^3.h^{-1}$)	Subsequent rate of chondrocyte volume decrease ($\mu m^3.h^{-1}$)
37 (control)	34.38 ± 0.03	2.87 ± 0.85
20	22.50 ± 0.23 **	0.56 ± 0.01 ***

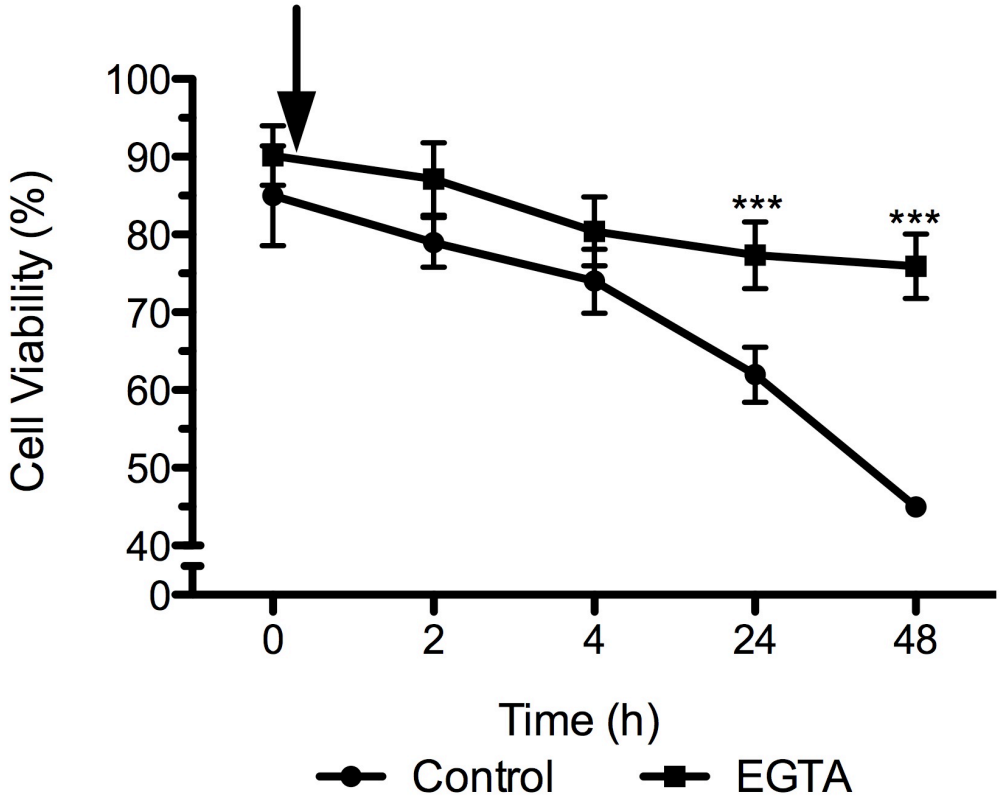
Figure 3.11: Mechanical impact induced decrease in cell volume is temperature dependent.

*Cartilage explants were kept at control 37°C or 20°C and subjected to a single mechanical impact. Samples were loaded with Calcein AM (5 μM) and visualised using CLSM prior to and at 2 h, 24 h and 48 h post mechanical impact. Isosurface volume analysis was used to quantify cell volume. Decreased temperature was observed to decrease the mechanical impact induced decrease in cell volume was seen. N=12 and n=60 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005 all vs. control.* Arrow denotes impact point.

3.2.6 The Role of Extracellular Calcium in Chondrocyte Death and IIVD

Calcium signalling is known to play a role in many aspects of mechanotransduction, including regulatory volume decrease (RVD). Calcium changes within the cell can be a result of release from intracellular stores, or flux from the extracellular environment via membrane channels. To investigate the possible role of calcium signalling in chondrocyte death and IIVD, samples were incubated with EGTA (2 mM), a calcium chelator, to remove extracellular calcium ($[Ca^{2+}]_0$) and thus reveal any involvement of calcium influx via membrane channels in chondrocyte death or IIVD.

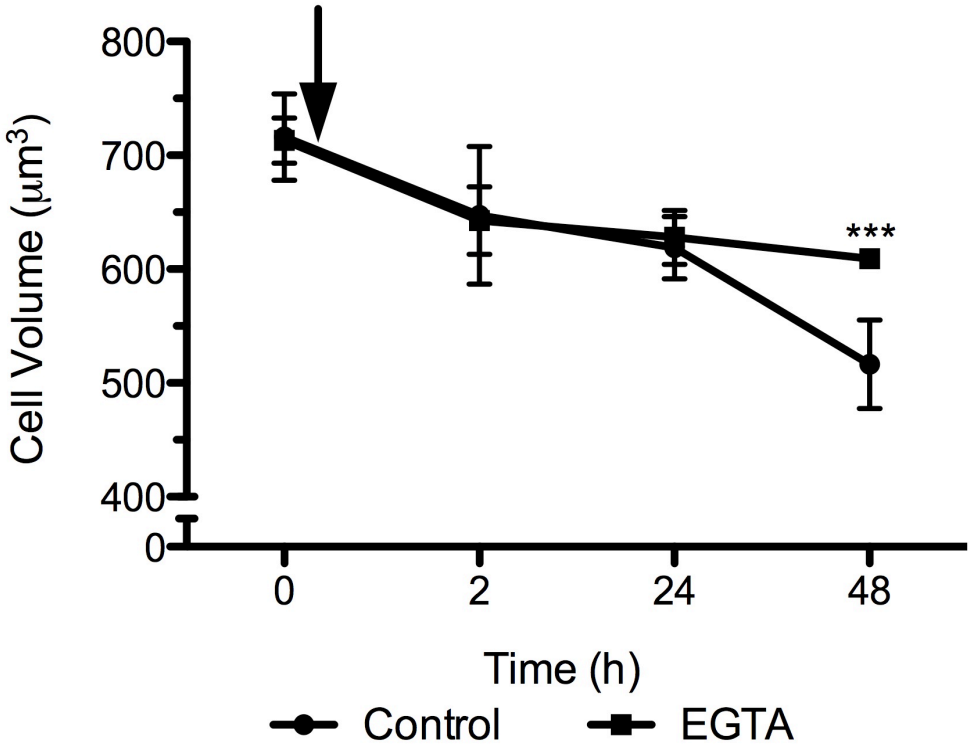
Removal of $[Ca^{2+}]_0$ by EGTA was observed to significantly decrease both the initial rate (2.34±0.15 $.h^{-1}$) and the subsequent rate (0.12±0.02 $\%.h^{-1}$) of cell death post mechanical impact when compared to control rates of 2.75±0.14 $\%.h^{-1}$ and 0.66±0.03 $\%.h^{-1}$ respectively. This resulted in a 1.7 fold increase in cell viability at 48 h compared to control (*Fig. 3.12*). $[Ca^{2+}]_0$ was further observed to have a role within the subsequent phase of IIVD, whereby the subsequent rate of IIVD was significantly decreased ($p<0.005$) to 0.73±0.13 $\mu m^3.h^{-1}$ (*Fig. 3.13*). Conversely, the removal of $[Ca^{2+}]_0$ was not observed to have a significant effect on the initial rate of IIVD.



Condition	Initial rate of chondrocyte death (%.h^{-1})	Subsequent rate of chondrocyte death (%.h^{-1})
Control	2.75 ± 0.14	0.66 ± 0.03
EGTA	2.34 ± 0.15 *	0.12 ± 0.02 ***

Figure 3.12: Cell death post impact is dependant on extracellular calcium.

*Cartilage explants were pre-treated for 1 h with control isotonic or EGTA supplemented (2 mM) conditions and subjected to a single mechanical impact. Samples were loaded with Calcein AM (5 μM) and PI (1 μM) and visualised using CLSM prior to and at 2 h, 24 h and 48 h post mechanical impact. Imaris spot analysis was used to quantify cell viability. Removal of extracellular calcium by EGTA was observed to protect against impact induced cell death. N=12 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005 all vs. control. Arrow denotes impact point.*



Condition	Initial rate of chondrocyte volume decrease ($\mu m^3.h^{-1}$)	Subsequent rate of chondrocyte volume decrease ($\mu m^3.h^{-1}$)
Control	34.38 ± 0.03	2.87 ± 0.85
EGTA	35.10 ± 0.04	0.73 ± 0.13 **

Figure 3.13: Mechanical impact induced decrease in cell volume is dependent of extracellular calcium.

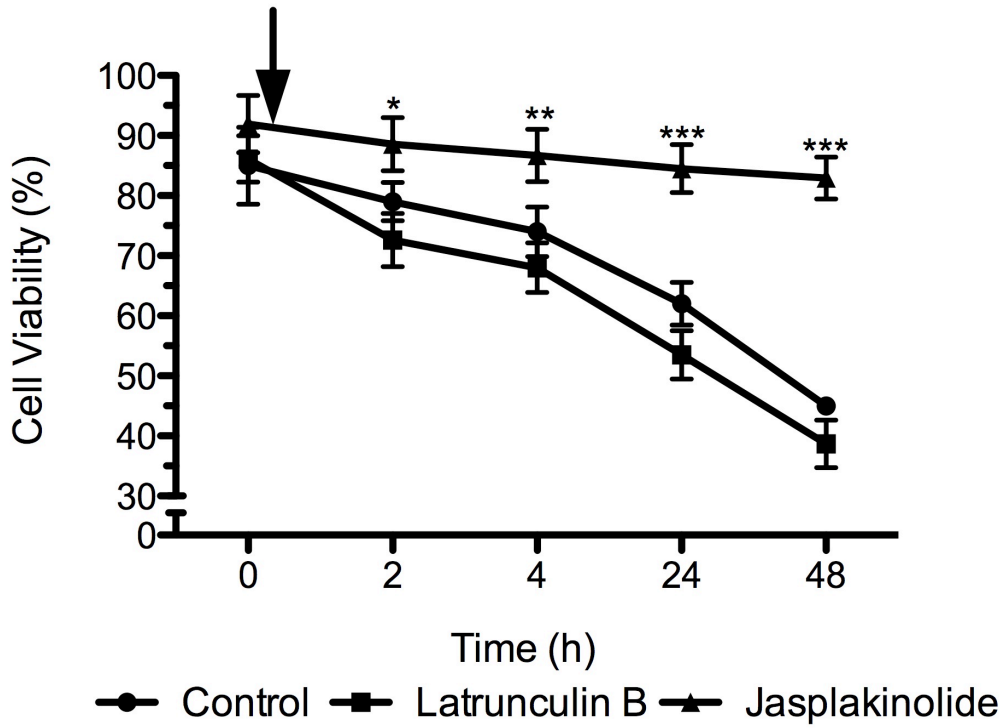
*Cartilage explants were pre-treated for 1 h with control isotonic or EGTA supplemented (2 mM) conditions and subjected to a single mechanical insult. Samples were stained with Calcein AM (5 μM) and visualised using CLSM prior to and at 2 h, 24 h and 48 h post mechanical impact. Isosurface volume analysis was used to quantify cell volume. Removal of extracellular calcium was observed to decrease the subsequent rate of mechanical impact induced decrease in cell volume. N=12 and n=60 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005 all vs. control. Arrow denotes impact point.*

3.2.7 The Role of IIAD in Chondrocyte Death Post Mechanical Impact

The F-actin cytoskeleton is essential to maintaining structural cell integrity (Blain, 2009, Guilak *et al*., 1995, Wang *et al*., 1993), evidenced by the defective function and decreased number of viable chondrocytes within OA tissue (Blain, 2009), thus the observed IIAD could have a role within impact induced cell death. Therefore, latrunculin B (10 μM), an inhibitor of actin polymerisation that disrupts actin filaments (Spector *et al*., 1989), and jasplakinolide (5 μM), a stabilizer of F-actin (Bubb *et al*., 2000), were employed to determine the effects of exacerbating and inhibiting IIAD respectively, on cell viability post mechanical impact.

Disrupting F-actin, and thus enhancing IIAD, via latrunculin B was observed to significantly ($p < 0.05$) increase chondrocyte death post mechanical impact, with a viability of 38.68±3.98 % at 48 h compared to a control viability of 45.01±0.99 %. Conversely, stabilising F-actin and thus blocking IIAD, via jasplakinolide was noted to be chondroprotective, with no significant chondrocyte death at 48 h (85.92±3.50 %; *Fig. 3.14*).

Latrunculin B and jasplakinolide had opposing effects of the rates of chondrocyte death, whereby latrunculin B increased the initial rate of cell death by 30 % and the subsequent rate by 8 %. Jasplakinolide, on the other hand, decreased the initial rate of chondrocyte death by 52 % and the subsequent rate by 88 %, thus establishing a role for IIAD within the mechanism of chondrocyte death post mechanical impact (*Fig. 3.14*).



Condition	Initial rate of chondrocyte death (%.h^{-1})	Subsequent rate of chondrocyte death (%.h^{-1})
Control	2.75 ± 0.14	0.66 ± 0.03
Latrunculin B	3.52 ± 0.34 **	0.71 ± 0.01 **
Jasplakinolide	1.31 ± 0.59 ***	0.08 ± 0.03 ***

Figure 3.14: Cell death post impact is dependant on F-actin depolymerisation.
*Cartilage explants were pre-treated for 1 h with control isotonic, Latrunculin B (10 μM) or Jasplakinolide supplemented (5 μM) conditions and subjected to a single mechanical impact. Samples were loaded with Calcein AM (5 μM) and PI (1 μM) and visualised using CLSM prior to and at 2 h, 24 h and 48 h post mechanical impact. Imaris spot analysis was used to quantify cell viability. Stabilising of f-actin by Jasplakinolide was observed to significantly reduce both the initial and subsequent rate of cell death post mechanical impact. Disruption of f-actin by Latrunculin B was observed not to affect cell death. N=12 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005 all vs. control. Arrow denotes impact point.*

3.3 Summary

Mechanical impact was observed to induce biphasic chondrocyte death over 48 h post impact. A rapid initial death was observed, followed by a slower subsequent death. Similarly, mechanical impact was seen to result in a biphasic IIVD, again a rapid initial decrease was observed followed by a slower subsequent decrease. IIAD was additionally observed post mechanical impact, although this was a rapid response, limited to the first 2 h post mechanical impact. Furthermore, mechanical impact resulted in increased pro and anti-inflammatory cytokines, pro-inflammatory cytokines peaking immediately post impact, and anti-inflammatory cytokines peaking at 24 h (*Table 3.1*).

Cell swelling induced by hypo-osmotic challenge increased both the initial and subsequent rates of cell death post mechanical impact, and additionally resulted in an increased initial and subsequent rate of IIVD. Furthermore, hypo-osmotic treatment decreased initial F-actin and but did not affect the rate of IIAD. Additionally, hypo-osmotic treatment was observed to increase both pro and anti-inflammatory cytokines, whilst not affecting the expression pattern of cytokine release. These data indicated cell swelling, such as that occurring within OA tissue, increases chondrocyte sensitivity to mechanical impact.

Cell shrinkage by hyperosmotic challenge was seen to have chondroprotective effects post mechanical impact. Hyperosmotic challenge additionally induced a decrease in both initial and subsequent rates of IIVD, and increase in F-actin prior to impact, and protected against IIAD. Furthermore, hyperosmotic treatment was seen to decrease pro and anti-inflammatory cytokines. These data imply a role for cell shrinkage in chondroprotection.

Decreasing tissue temperature was observed to protect against cell death post mechanical impact, decreasing the subsequent rate of chondrocyte death. Decreased temperature additionally decreased both the initial and

subsequent rates of IIVD, indicating that IIVD and the secondary phase of impact induced cell death are active processes.

Similarly, removal of $[Ca^{2+}]_0$ with EGTA was chondroprotective, decreasing both the initial and subsequent rates of chondrocyte death. However, removal of $[Ca^{2+}]_0$ only decreased the secondary phase of IIVD, indicating that whilst the initial phase of IIVD is independent of calcium influx via membrane channels, the secondary phase is not.

Finally, simulation of IIAD with latrunculin B was observed to exacerbate cell death, whereas prevention of IIAD with jasplakinolide was observed to be chondroprotective, indicating a role for IIAD cell death post mechanical impact.

	Mechanical impact induced responses
Viability	Biphasic cell death
Volume	Biphasic IIVD
F-actin	Rapid IIAD
Cytokines	Increased, pro-inflammatory peak at 0h, anti-inflammatory peak at 24 h

Table 3.1: Articular cartilage responses to single mechanical impact

Summary of the cellular responses of bovine articular cartilage when subjected to a single mechanical impact.

Chapter 4: Chondroprotective Actions of Nutritional Supplements and Pharmaceuticals

4.1 Introduction

Mechanical loading, whilst integral to maintaining healthy cartilage, at abnormal or traumatic levels induces a decrease in viable cells, extracellular matrix (ECM) degradation and actin destabilisation (Oliveria *et al*., 1999, Tew *et al*., 2000). As previously observed (*Chapter 3*), single mechanical trauma stimulated significant biphasic chondrocyte death, biphasic trauma induced volume decrease (IIVD) and rapid trauma induced actin decrease (IIAD) in addition to inducing increased inflammatory cytokine production. Previous studies have reported mechanical impact to result in ECM degradation *via* a decrease in proteoglycan (PG) and collage II synthesis, and an increase in catabolic proteases such as matrix metalloproteinases (MMPs). Additionally, pro-inflammatory cytokines, including IL-1β have been noted to induce chondrocyte death and stimulate ECM degradation (Liu *et al*., 1994, Rowan and Young, 2007, Westacott and Sharif, 1996).

Osteoarthritis (OA) is characterised by a loss of viable chondrocytes and ECM degeneration, thus the cellular and ECM effects resultant of mechanical impact are indicated as severe risk factors for the development of OA. It is therefore of interest to investigate protective mechanisms against the effects of mechanical impact, namely chondrocyte death, IIVD, IIAD and inflammation. In recent years, promising chondroprotective agents evaluated in clinical trials include risedronate, a biphosphonate approved for the treatment of osteoporosis (Van Offel *et al*., 2002, Van Offel *et al*., 2005), and doxycycline, an antibiotic (Smith *et al*., 1996, Shlopov *et al*., 1999), though neither has been associated with definitive results (Felson and Kim, 2007). Thus, alternative agents with possibilities of chondroprotective actions were studied here, including RVD inhibitors; REV 5901 and tamoxifen, and nutriceuticals; chondroitin sulphate and glucosamine sulphate.

4.1.1 Chondroprotective Agents

REV 5901 is an antagonist of cysteinyl-leukotriene receptors (a family of G-protein coupled receptors), in porcine epithelial cells (Van Inwegen *et al*., 1987), an inhibitor of 5-lipoxygenase (Musser *et al*., 1987) and an inhibitor

of regulatory volume decrease (RVD) in articular chondrocytes (Hall and Bush, 2001). Given the effect of REV 5901 on RVD, and that IIVD is a consequence of mechanical impact, REV 5901 was investigated as a possible chondroprotective agent.

Tamoxifen, a tissue-dependent oestrogen receptor agonist/antagonist, is routinely used as a cancer treatment, primarily in oestrogen sensitive breast cancer. However, it is also know to be an inhibitor of volume-sensitive organic anion channels (VSOACs) and thus an inhibitor of RVD, as the postulated RVD osmolyte channel is known to be VSOAC similar (Hall, 1995). Additionally, tamoxifen has anticalmodulin actions, suggesting it could have a role in actin regulation as calmodulin is an upstream molecule necessary for the expression of cofilin, an actin depolymerising protein (Ono, 2007). With IIAD and IIVD features of mechanical impact, tamoxifen has the potential for chondroprotective actions.

Chondroitin sulphate, a high molecular weight glycosaminoglycan (GAG), constitutes the majority of GAGs found within the ECM, branched from PGs. Chondroitin sulphate is thus considered essential for the structural integrity of the tissue (Kelly, 1998, Lippiello *et al*., 2000). It has additionally been reported that chondroitin sulphate stimulates cartilage repair, inhibits catabolic enzymes and has anti-inflammatory actions (Huskisson, 2008, Deal and Moskowitz, 1999), thus indicating a possible use as a chondroprotective agent.

Glucosamine sulphate, a monosaccharide, is the principle substrate in the synthesis of PGs (Simanek *et al*., 2005). Loss of PGs within the ECM is a characteristic of both mechanical impact and OA, thus it is hypothesised that glucosamine sulphate could have chondroprotective mechanisms by providing increased potential for PG synthesis (Huskisson, 2008, Simanek *et al*., 2005, Pavelka *et al*., 2002).

4.1.2 Chapter Aims

This study aims to investigate the chondroprotective potential of nutriceuticals and pharmaceuticals.

Specifically by:

1. Study of the effects of named nutriceuticals and pharmaceuticals post mechanical impact on chondrocyte viability, volume and the actin cytoskeleton.
2. Determination of the effects of named nutriceuticals and pharmaceuticals on inflammatory cytokine concentrations in articular cartilage post mechanical impact.
3. Investigation of the involvement of a known calcium signalling pathway in the potential mechanism of chondroprotection of named nutriceuticals and pharmaceuticals.

4.2 Results

4.2.1 REV 5901: Chondroprotective Effects

REV 5901 has been previously shown to have effects on mechanotransduction pathways, whereby it is an inhibitor of regulatory volume decrease (Bush and Hall, 2001a). Given the effect of REV 5901 on RVD, and that IIVD has been observed here to be a consequence of mechanical impact, REV 5901 was investigated as a possible chondroprotective agent. Before further investigating the potential chondroprotective mechanisms of REV 5901, it was first necessary to confirm the effects of both REV 5901 and DMSO, the vehicle of REV 5901, did not alter DMEM osmolarity and thus simply exert osmotic effects.

The initial osmolarity of DMEM was confirmed using a Vapro™ vapour pressure osmometer to be 280.10±0.75 mOsm which did not significantly change ($P>0.05$) either by the addition of REV5901 or the DMSO vehicle whereby the osmolarities were 282.30±1.23 mOsm and 283.21±2.36 mOsm respectively (*Fig. 4.1*).

Having determined the effects of REV 5901 on cell volume to be independent of osmotic changes, the influence of REV 5901 on cell volume was investigated on isotonic and hyperosmotic samples as a model of 'evening' cartilage. REV 5901 was observed to significantly decrease chondrocyte volume, regardless of initial volume. Whereby, isotonic samples (*Fig. 4.2A*), with a mean volume of 716.14±37.81 μm^3, exhibited a decrease in cell volume post incubation with REV 5901 (*Fig. 4.2B*) to 426.29 μm^3. Similarly, hyperosmotic samples (*Fig. 4.2C*), with a mean volume of 561.46±25.49 μm^3, further decreased in volume post incubation with REV 5901 (*Fig. 4.2.D*) to 379.19±13.63 μm^3 (*Fig. 4.2E*).

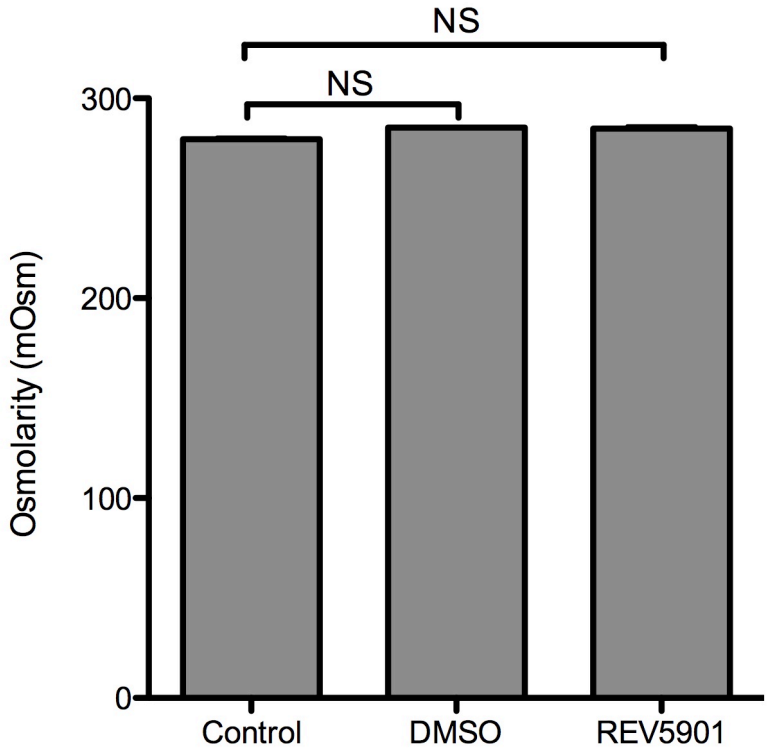


Figure 4.1: The addition of REV 5901 has no effect on medium osmolarity.

Samples of control isotonic, DMSO (0.1 %) and REV 5901 (5 μM) supplemented DMEM were measured for osmolarity using Vapro ™ vapour pressure osmometer. Neither DMSO or REV 5901 was observed to significantly alter medium osmolarity. N=25

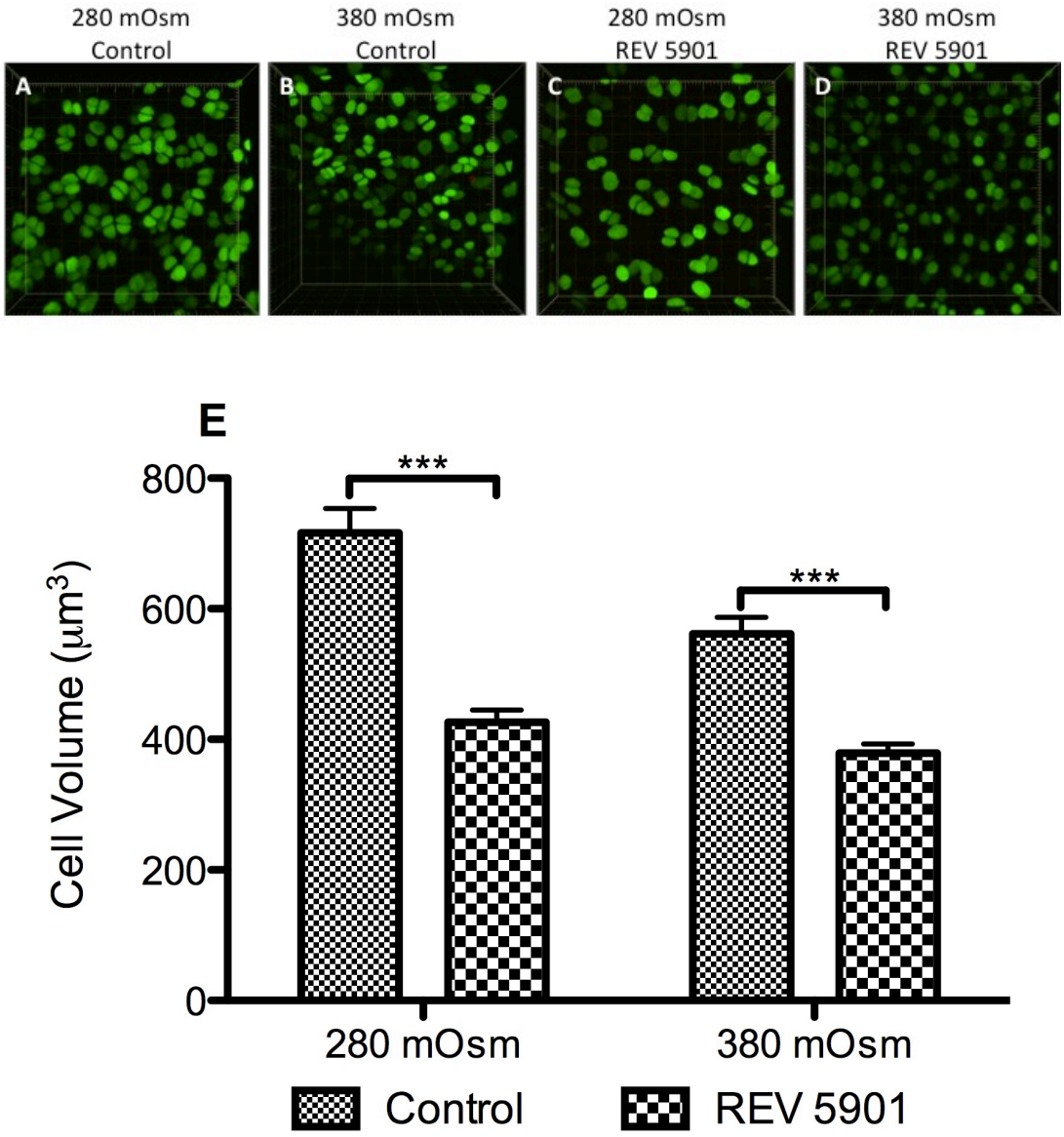


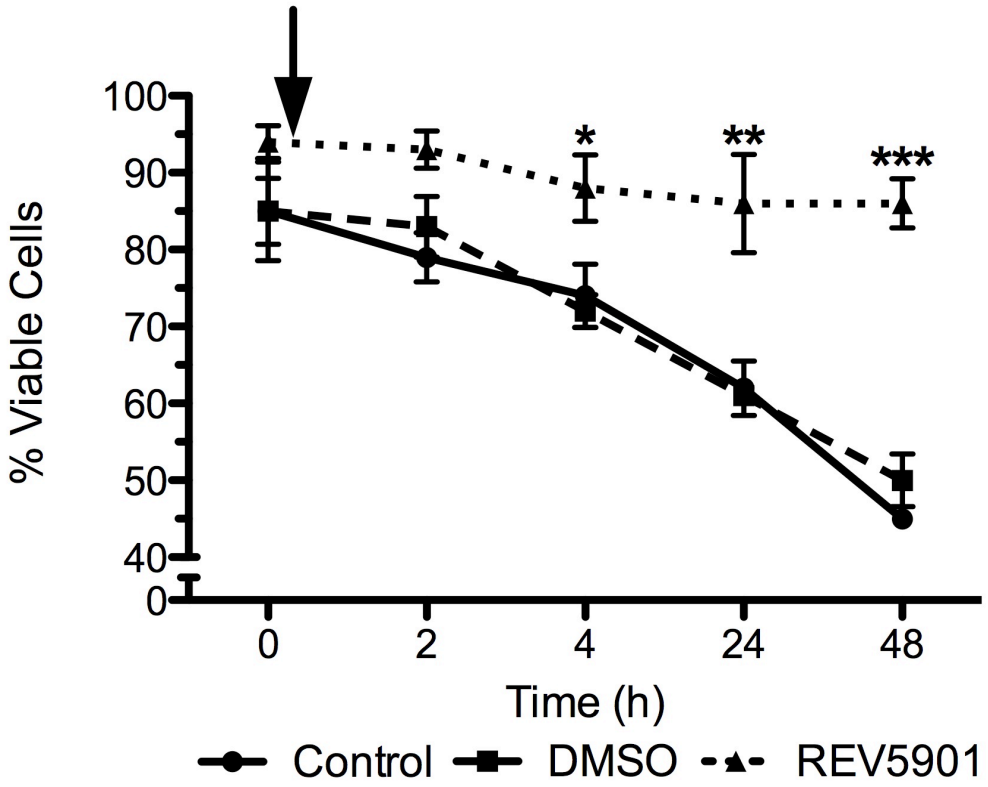
Figure 4.2: REV 5901 decreases cell volume post osmotic challenge.

*Cartilage explants were pre-treated for 1 h with control isotonic or hyperosmotic (380 mOsm) DMEM and for a subsequent 1 h with control isotonic (280 mOsm; A&B) or REV 5901 (50 μM; C&D) supplemented DMEM. Samples were loaded with Calcein AM (5 μM) and visualised using CLSM immediately post treatment. Isosurface volume analysis was used to quantify cell volume. REV 5901 was observed to significantly decrease cell volume regardless of previous osmotic challenge (E). N=12 and n=60 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005.*

The effects of REV 5901 on chondrocyte volume further supported the potential for REV 5901 as a chondroprotective agent, thus to investigate REV 5901 as a chondroprotective agent samples were pre-treated with control (280 mOsm), DMSO (0.1 %) or REV 5901 (50 μM) supplemented DMEM and analysed post mechanical impact by CLSM for viability and volume.

REV 5901 was initially confirmed to have no adverse effects of cell viability, with a pre-impact viability of 94.08±2.12 $\%.h^{-1}$. REV 5901 treatment resulted in a decrease in both the initial (1.50±0.58 $\%.h^{-1}$) and subsequent (0.04±0.03 $\%.h^{-1}$) rate of cell death compared to control (Initial: 2.75±0.14 $\%.h^{-1}$, Subsequent: 0.66±0.03 $\%.h^{-1}$). This resulted in no significant change in cell viability post mechanical impact (*Fig. 4.3*), conversely, DMSO had no significantly different effects to control conditions, thus demonstrating the chondroprotective effects of REV 5901 where not an artefact of the DMSO vehicle.

Furthermore, REV 5901 treatment was seen to significantly decrease cell volume ($p<0.005$) by 27 % prior to mechanical impact to 552.68±27.26 μm^3 when compared to control samples (716.14±35.82 μm^3; *Fig. 4.4C&D*). Biphasic IIVD was observed in REV 5901 conditions post mechanical impact, however both the initial (20.53±0.03 $\mu m^3.h^{-1}$) and the subsequent (0.79±0.06 $\mu m^3.h^{-1}$) rate of IIVD were observed to be significantly slower when compared to control conditions (initial: 34.38±0.03 $m^3.h^{-1}$, subsequent: 2.87±0.85 $m^3.h^{-1}$; *Fig. 4.5*). DMSO conditions did not significantly ($p>0.05$) alter initial cell volume (*Fig. 4.4B*) or rates of IIVD, again confirming the actions of REV 5901 to be solely due to REV 5901 as such, not the DMSO vehicle.



Condition	Initial rate of chondrocyte death (%.h^{-1})	Subsequent rate of chondrocyte death (%.h^{-1})
Control	2.75 ± 0.14	0.66 ± 0.03
DMSO	3.25 ± 1.29	0.59 ± 0.05
REV 5901	1.50 ± 0.58 ***	0.04 ± 0.03 ***

Figure 4.3: REV 5901 pre-treatment protects against cell death post impact.

*Cartilage explants were pre-treated for 1 h with control isotonic, DMSO or REV 5901 (50 μM) supplemented DMEM and subjected to a single mechanical impact. Samples were loaded with Calcein AM (5 μM) and PI (1 μM) and visualised using CLSM prior to and at 2 h, 4 h, 24 h and 48 h post mechanical impact. Imaris spot analysis was used to quantify cell viability. Treatment with REV 5901 was seen to protect against cell death. N=12 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005 all vs. control. Arrow denotes impact point.*

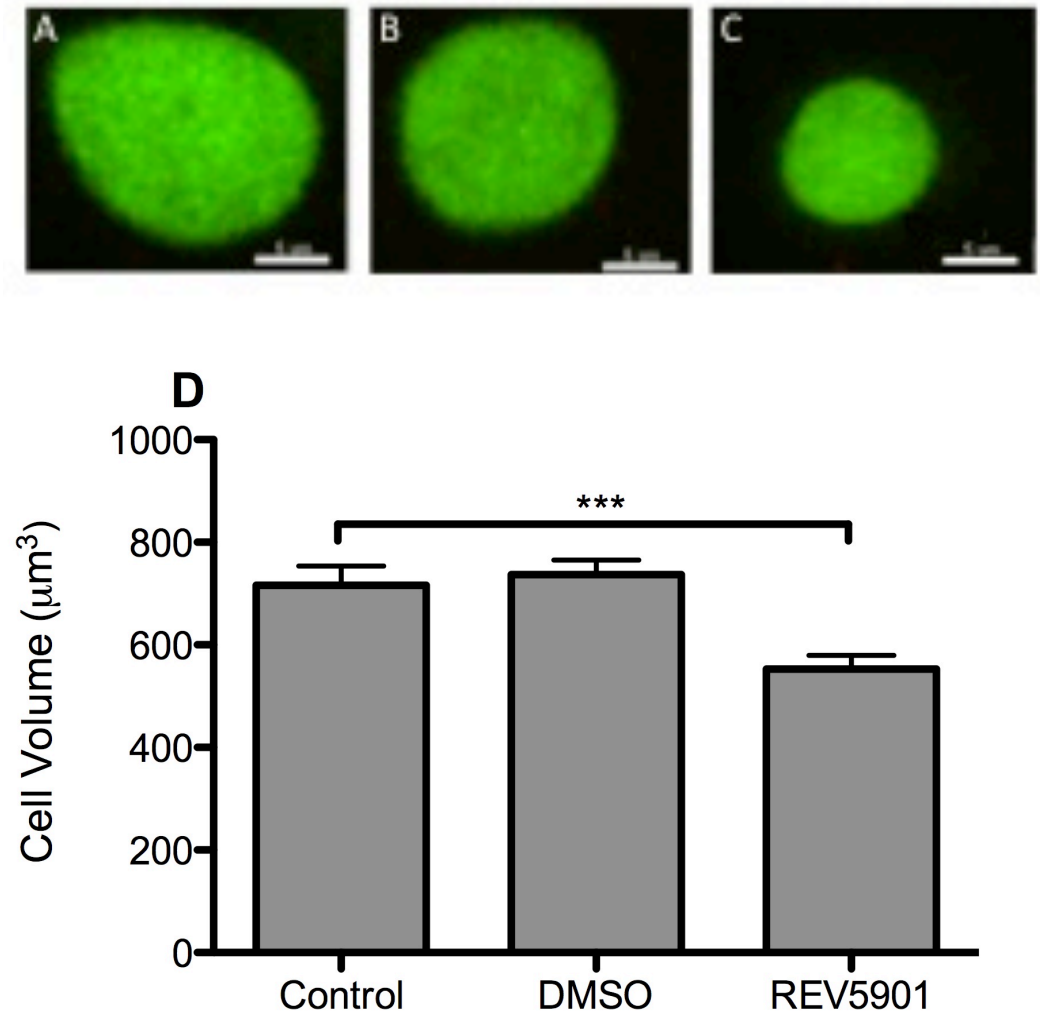
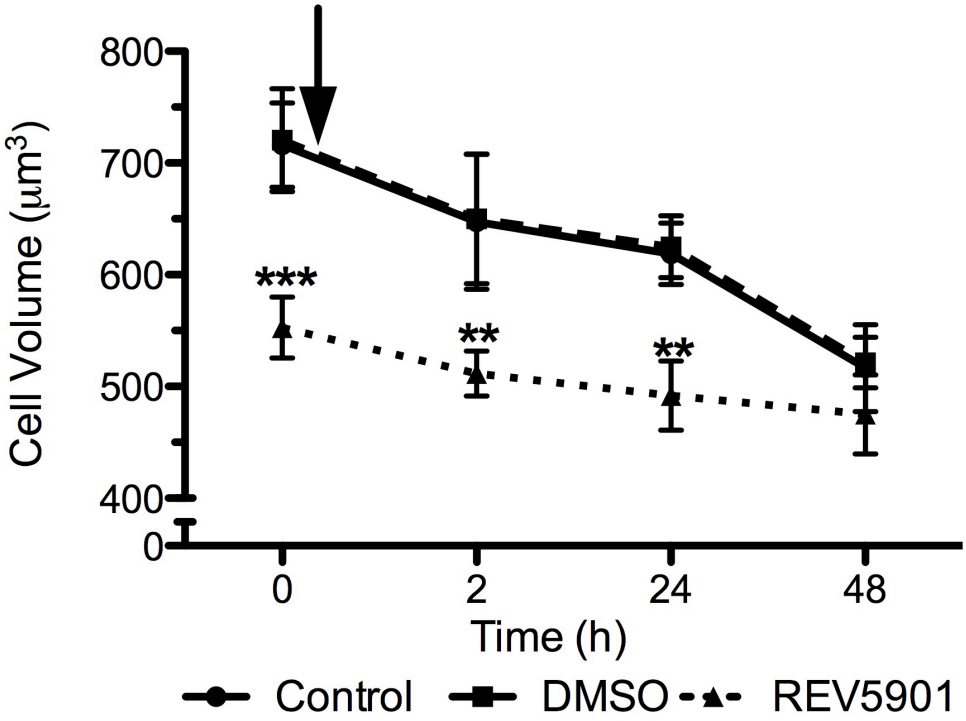


Figure 4.4: REV 5901 decreases chondrocyte volume.

*Cartilage explants were pre-treated for 1 h with control isotonic (A), DMSO (B) or REV 5901 (C; 50 μM) supplemented DMEM and subjected to a single mechanical impact. Samples were loaded with Calcein AM (5 μM) and visualised using CLSM prior to mechanical impact. Isosurface volume analysis was used to quantify cell volume. REV 5901 significantly decreased cell volume prior to mechanical impact (D). N=12 and n=60 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005 all vs. control.*

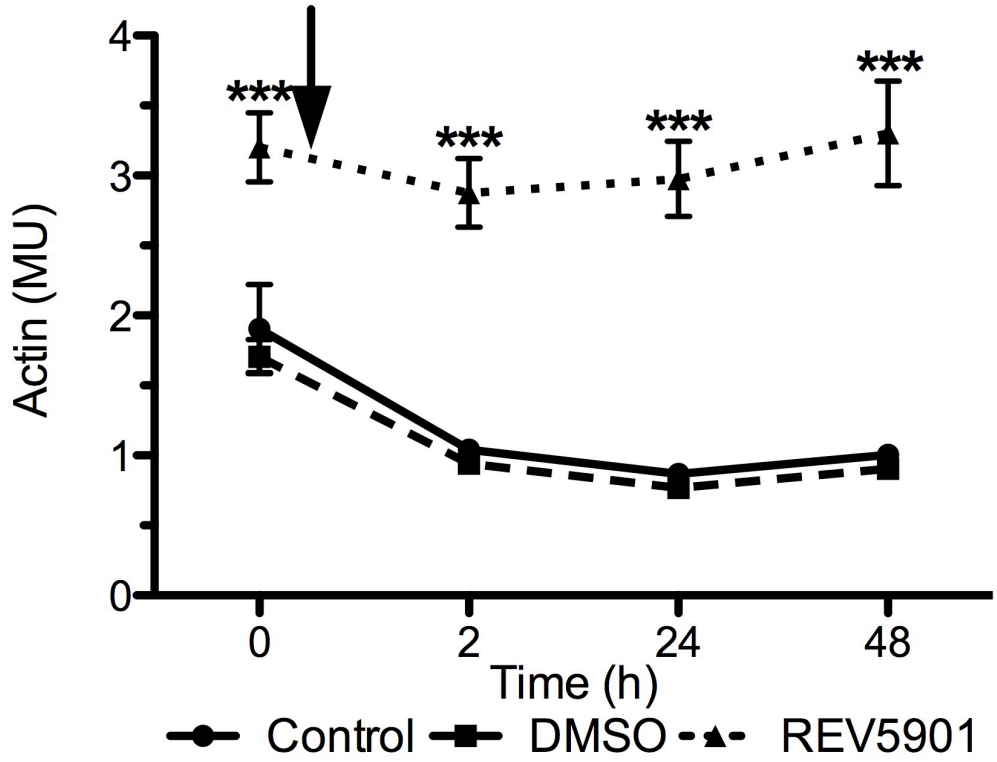


Condition	Initial rate of chondrocyte volume decrease ($\mu m^3.h^{-1}$)	Subsequent rate of chondrocyte volume decrease ($\mu m^3.h^{-1}$)
Control	34.38 ± 0.03	2.87 ± 0.85
DMSO	35.30 ± 0.14	2.82 ± 0.19
REV 5901	20.53 ± 0.03 ***	0.79 ± 0.06 ***

Figure 4.5: REV 5901 decreases mechanical impact induced volume decrease.
*Cartilage explants were pre-treated for 1 h with control isotonic, DMSO or REV 5901 (50 μM) supplemented DMEM and subjected to a single mechanical impact. Samples were loaded with Calcein AM (5 μM) and visualised using CLSM prior to and at 2 h, 4 h, 24 h and 48 h post mechanical impact. Isosurface volume analysis was used to quantify cell volume. REV 5901 treated samples were observed to have a significantly slower initial and subsequent rate of volume decrease. N=12 and n=60 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005 all vs. control. Arrow denotes impact point.*

Further, the role of the F-actin cytoskeleton in the chondroprotective effects of REV 5901 was investigated using linear profiling and the PQ Factor, as previously described (*See Methods and Materials*).

REV 5901 was observed to increase F-actin prior to mechanical impact by 68 % to 3.20±0.24 MU compared to control values of 1.91±0.32 MU (*Fig. 4.6*). Additionally, REV 5901 treatment induced a decrease in the rate of IIAD from a control rate of 0.43±0.03 $MU.h^{-1}$ to 0.16±0.03 $MU.h^{-1}$, thus resulting in no significant change ($p>0.05$) in F-actin from prior to impact (3.20±0.24 MU) to 48 h post mechanical impact (3.30±0.37 MU; *Fig. 4.6*). DMSO was noted to not alter F-actin prior to impact, or alter the rate of IIAD observed in control samples, further confirming the chondroprotective effects of REV 5901 as independent of the DMSO vehicle.



Condition	Initial rate of actin polymerisation ($MU.h^{1}$)	Subsequent rate of actin polymerisation ($MU.h^{-1}$)
Control	-0.43 ± 0.03	0.00 ± 0.00
DMSO	-0.38 ± 0.06	0.00 ± 0.00
REV 5901	-0.16 ± 0.03 ***	0.01 ± 0.02

Figure 4.6: REV 5901 protects against mechanical impact induced F-actin decrease. *Cartilage explants were pre-treated for 1 h with control isotonic, DMSO or REV 5901 (50 μM) supplemented DMEM and subjected to a single mechanical impact. Samples were fixed with 4 % paraformaldehyde and stained with Alexa Fluor 488 phalloidin (5 μl/ml). Cells were visualised using CLSM prior to and at 2 h, 24 h and 48 h post mechanical impact. Linear profiling analysis was used to quantify f-actin, expressed relative to volume changes. REV 5901 was observed to significantly increase f-actin prior to mechanical impact and significantly decrease the rate of mechanical impact induced f-actin decrease. N=12 and n=60 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005 all vs. control. Arrow denotes impact point.*

REV 5901 is a known inhibitor of 5-lipoxygenase (5-LO) (Musser *et al*., 1987), thus to confirm the presence of 5-LO within articular cartilage, and the inhibitory effects of REV 5901, LTB_4, a downstream leukotriene of 5-LO, concentration was analysed by ELISA. LTB_4 concentration was observed to increase almost 4 fold in response to mechanical impact (3.72±0.19 AU), confirming the presence of 5-LO within articular cartilage. REV 5901 was noted to significantly decrease LTB_4 by 92 % post mechanical impact, and by 70 % when compared to non-impacted controls, to 0.30±0.01 AU (*Fig. 4.7*). Thus, the inhibitory effects of REV 5901 on 5-LO were confirmed.

Leukotrienes, such as LTB_4, have been noted to be involved in the production of inflammatory cytokines in synovial cells (Kageyama *et al*., 1994), thus the possible anti-inflammatory effects of REV 5901 were investigated post mechanical impact, whereby supernatant samples were collected immediately and at 2, 24 and 48 h post mechanical impact and analysed by ELISA for cytokine concentration as previously described (*See Methods and Materials*).

REV 5901 was observed to decrease pro-inflammatory cytokine production immediately post impact, decreasing IL-1β by 87 % to 0.75±0.09 AU (*Fig. 4.8A*) and MCP-1 by 54 % to 0.67±0.11 AU (*Fig. 4.8C*). In addition, REV 5901 decreased anti-inflammatory cytokine release, whereby the peak IL-10 concentration observed at 24 h in control samples was reduced by 84 % to 0.90±0.17 AU (*Fig. 4.8B*). Furthermore, REV 5901 was observed to prevent any impact induced increase in inflammatory cytokines, with no significant ($p>0.05$) changes in cytokine concentration observed over 48 h post impact (*Fig. 4.8*).

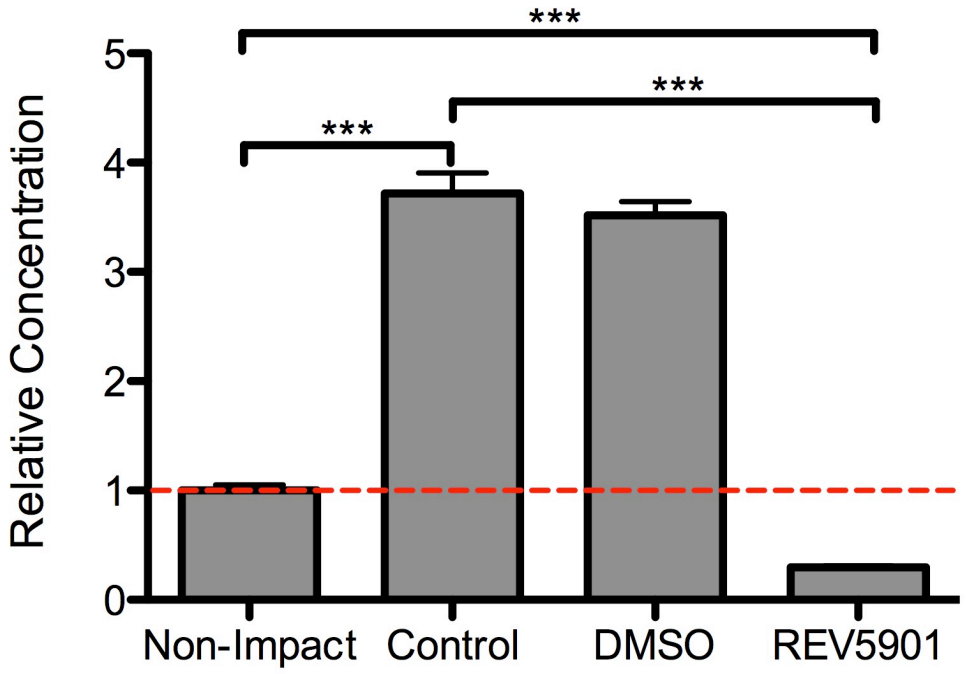


Figure 4.7: REV 5901 decreases LTB_4 concentration in articular cartilage.

*Cartilage explants were pre-treated for 1 h with control isotonic, DMSO or REV 5901 (50 μM) supplemented DMEM and subjected to a single mechanical impact. Supernatant at 4 h post mechanical impact was analysed by ELISA and LTB_4 concentration expressed relative to non-impacted control samples. LTB_4 concentration was seen to significantly increase post mechanical impact. Conversely, pre-treatment with REV 5901 was observed to significantly decrease LTB_4 concentration compared to both control and non-impacted control. N=12 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005.*

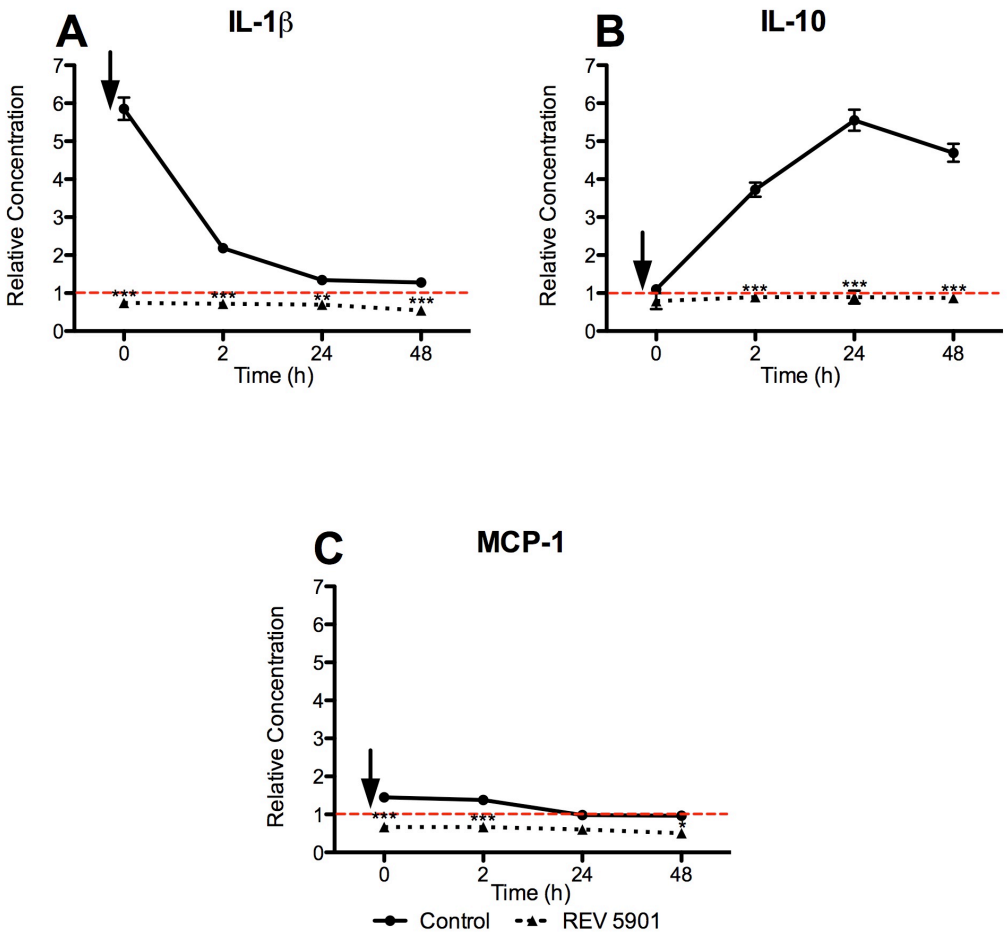


Figure 4.8: REV 5901 decreases inflammatory cytokines post-mechanical impact.
*Cartilage explants were pre-treated for 1 h with control isotonic, DMSO or REV 5901 (50 μM) supplemented DMEM and subjected to a single mechanical impact. Supernatant samples immediately following and at 2 h, 24 h and 48 h post mechanical impact were analysed by ELISA and cytokine concentration expressed relative to non-impacted control samples. REV 5901 was observed to significantly decrease IL-1β (A), IL-10 (B) and MCP-1 (C) over the whole time course when compared to both control and non-impacted controls. N=12 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005. Arrow denotes impact point.*

4.2.2 The Role of Calcium Signalling in the Mechanism of Action of REV 5901

REV 5901 is a known inhibitor of RVD, whereby REV 5901 prevents a return to resting volume in *in situ* chondrocytes post hypo-osmotic challenge (Bush and Hall, 2001a). These actions have been previously observed within the laboratory to be dependent on intracellular calcium signalling, via the PKC/PLCβ_3 pathway (Qusous *et al*., 2011). Thus, the role of this pathway within the chondroprotective actions of REV 5901 demonstrated here was investigated, whereby samples were pre-incubated rottlerin (100 μM), an inhibitor of PKC, U73122 (100 μM), an antagonist of PLCβ_3 or EGTA (2mM), a calcium chelator, prior to incubation with REV 5901 (50 μM). Subsequent to mechanical impact, samples were analysed by CLSM for viability and volume and F-actin.

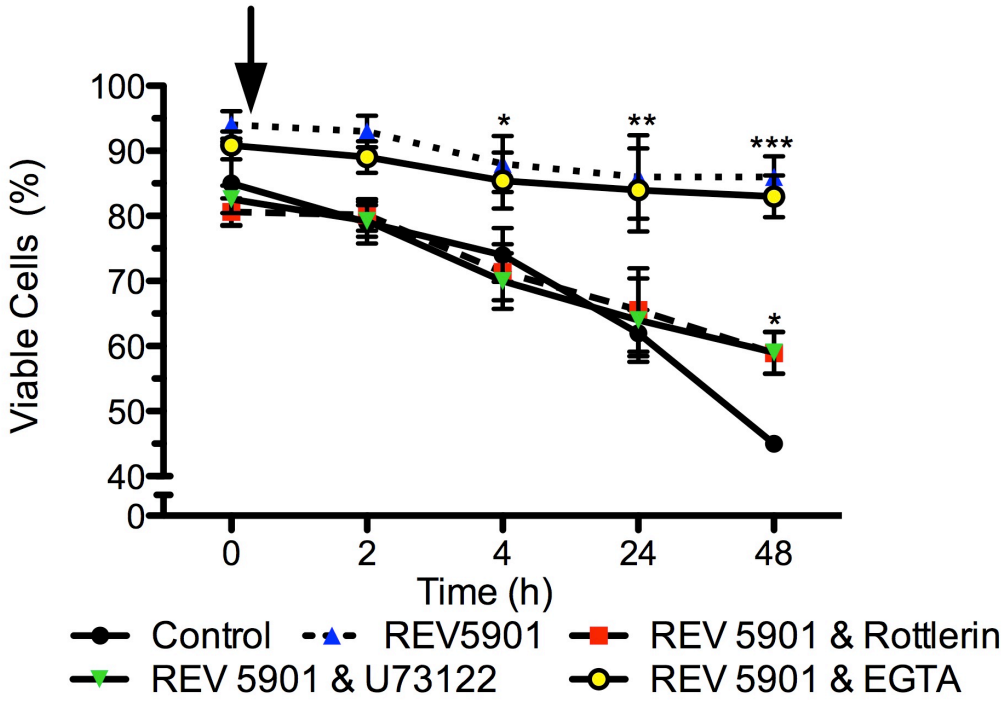
Rottlerin and U73122 both reversed the chondroprotective effects of REV 5901 post mechanical impact, whereby rottlerin and U73122 pre-incubation decreased chondrocyte viability at 48 h post impact by a further 27 %, compared to REV 5901 alone (*Fig. 4.9*). To this end, rottlerin and U73122 pre-incubation increased the initial rate of cell death to 2.31±0.21 %.h^{-1} and 3.13±0.85 %.h^{-1} respectively, values not significantly ($p>0.05$) different to control (2.75±0.14 %.h^{-1}). Conversely, EGTA pre-incubation did not affect the chondroprotective actions of REV 5901, suggesting REV 5901 acts via intracellular calcium signalling, not extracellular calcium flux (*Fig. 4.9*).

Furthermore, whilst REV 5901 was observed to decrease cell volume prior to mechanical impact to 552.68±27.26 μm^3, pre-incubation with rottlerin and U73122 prevented this decrease, displaying cell volumes of 680.89±17.34 μm^3 and 670.92±11.25 μm^3 respectively, not significantly different from control volumes of 716.14±37.81 μm^3 (*Fig 4.10*). Interestingly, rottlerin and U73122 were observed to block the initial rate IIVD, compared to both control (34.38±0.03 μm^3.h^{-1}) and REV 5901 (20.53±0.03 μm^3.h^{-1}) to 3.00±0.03 μm^3.h^{-1} and 2.50±0.41 μm^3.h^{-1} respectively (*Fig. 4.10*), indicating a role for the PKC/PLCβ_3 pathway within the IIVD mechanism. Removal of

extracellular calcium by EGTA was again observed to have no effect on the actions of REV 5901 or on IIVD.

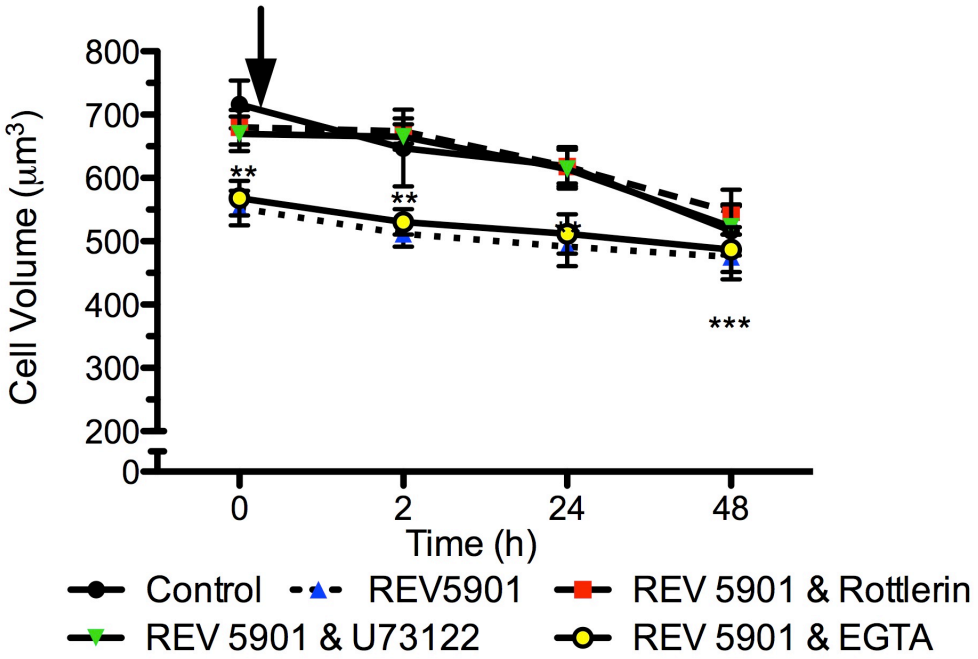
When studying F-actin responses, it was noted that rottlerin and U73122 both inhibited the REV 5901 induced increase in F-actin prior to mechanical impact, by 40 % and 30 % respectively (*Fig 4.11*). Furthermore, rottlerin and U73122 were seen to restore the observation of IIAD, whereby a decrease in F-actin was observed from 0 - 2 h post impact, not significantly different ($p>0.05$) from control (0.43 ± 0.03 $MU.h^{-1}$), of 0.46 ± 0.03 $MU.h^{-1}$ and 0.52 ± 0.01 $MU.h^{-1}$ respectively (*Fig. 4.11*). Conversely, EGTA exhibited an increase in F-actin prior to mechanical impact, not significantly different ($p>0.05$) to that observed with REV 5901, and did not exhibit IIAD (*Fig. 4.11*).

The role of the PKC/PLβ_3 pathway was additionally investigated in the anti-inflammatory effects of REV 5901. Neither rottlerin, U73122 nor EGTA were observed to inhibit the anti-inflammatory effects of REV 5901, whereby upon pre-incubation with rottlerin, U73122 and EGTA, pro-inflammatory cytokines IL-1β (*Fig. 4.12A*) and MCP-1 (*Fig. 4.12C*) remained decreased below non-impact control concentrations for 48 h post impact. Additionally, the decreased expression of IL-10 (*Fig. 4.12B*) induced by REV 5901 was maintained upon pre-incubation with rottlerin, U73122 and EGTA. These data suggest that the anti-inflammatory effects of REV 5901 are not contributed to by the PKC/PLCβ_3 signalling pathway.



Condition	Initial rate of chondrocyte death (%.h^{-1})	Subsequent rate of chondrocyte death (%.h^{-1})
Control	2.75 ± 0.14	0.66 ± 0.03
REV 5901	1.50 ± 0.58 ***	0.04 ± 0.03 ***
REV 5901 & Rottlerin	2.31 ± 0.21	0.28 ± 0.00 **
REV 5901 & U73122	3.14± 0.85	0.25 ± 0.03 **
REV 5901 & EGTA	1.35 ± 0.26 ***	0.05 ± 0.01 ***

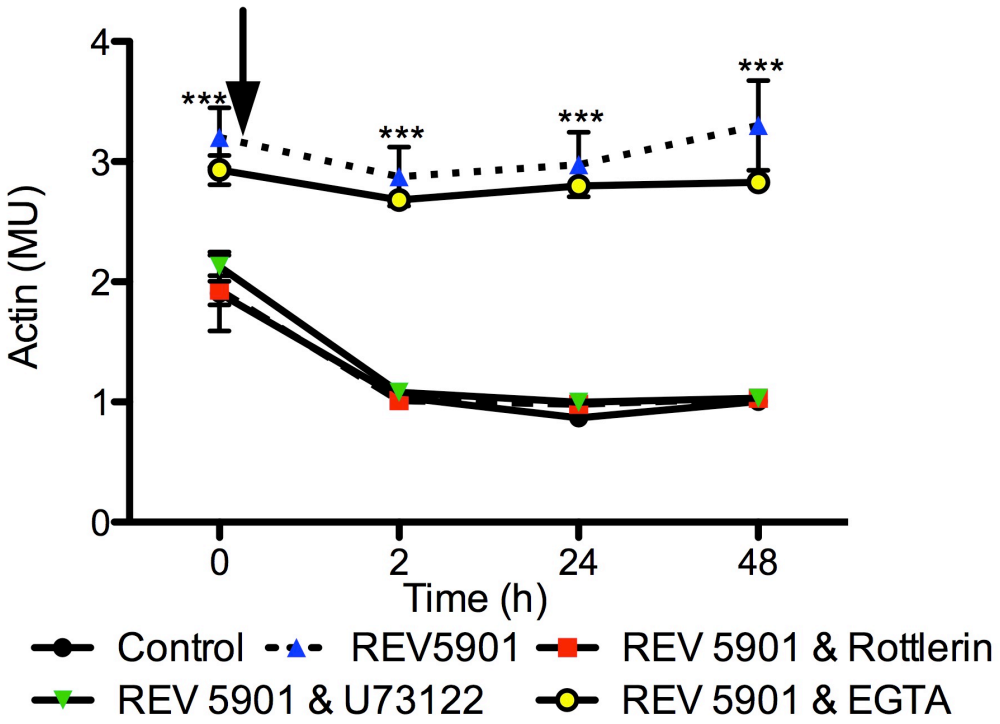
Figure 4.9: REV 5901's chondroprotective actions are PKC/PLCβ_3 dependent.
*Cartilage explants were pre-treated for 1 h with control isotonic, REV 5901 (50 μM), REV 5901 and rottlerin (100 μM), REV 5901 and U73122 (100 μM) or REV 5901 and EGTA (2mM) supplemented DMEM and subjected to a single mechanical impact. Samples were loaded with Calcein AM (5 μM) and PI (1 μM) and visualised using CLSM prior to and at 2 h, 4 h, 24 h and 48 h post mechanical impact. Imaris spot analysis was used to quantify cell viability. Treatment with rottlerin and U73122 was observed to reverse the chondroprotective effects of REV 5901. N=12 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005 all vs. control. Arrow denotes impact point.*



Condition	Initial rate of chondrocyte volume decrease ($\mu m^3.h^{-1}$)	Subsequent rate of chondrocyte volume decrease ($\mu m^3.h^{-1}$)
Control	34.38 ± 0.03	2.87 ± 0.85
REV 5901	20.53 ± 0.03 ***	0.79 ± 0.06 ***
REV 5901 & Rottlerin	3.00 ± 0.03 ***	2.79 ± 0.12
REV 5901 & U73122	2.50± 0.41 ***	3.01 ± 0.30
REV 5901 & EGTA	18.69 ± 0.56 ***	0.95 ± 0.02 ***

Figure 4.10: REV 5901 decreases cell volume by a PKC/PLCβ_3 dependant mechanism.

*Cartilage explants were pre-treated for 1 h with control isotonic, REV 5901 (50 μM), REV 5901 and rottlerin (100 μM), REV 5901 and U73122 (100 μM) or REV 5901 and EGTA (2mM) supplemented DMEM and subjected to a single mechanical impact. Samples were loaded with Calcein AM (5 μM) and visualised using CLSM prior to and at 2 h, 24 h and 48 h post mechanical impact. Isosurface volume analysis was used to quantify cell volume. Treatment with rottlerin and U73122 was observed to reverse the decrease in cell volume seen with REV 5901. Additionally, treatment with rottlerin and U73122 was observed to significantly decrease the initial rate of impact induced cell volume decrease compared to control. N=12 and n=60 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005 all vs. control Arrow denotes impact point.*



Condition	Initial rate of actin polymerisation ($MU.h^{-1}$)	Subsequent rate of actin polymerisation ($MU.h^{-1}$)
Control	-0.43 ± 0.03	0.00 ± 0.00
REV 5901	-0.16 ± 0.03 ***	0.01 ± 0.02
REV 5901 & Rottlerin	-0.46 ± 0.03	0.00 ± 0.00
REV 5901 & U73122	-0.52± 0.01	0.00 ± 0.00
REV 5901 & EGTA	-0.13 ± 0.01 ***	0.00 ± 0.00

Figure 4.11: REV 5901's protection against mechanical impact induced decrease in F-actin is PKC/PLCβ_3 dependant.

*Cartilage explants were pre-treated for 1 h with control isotonic, REV 5901 (50 μM), REV 5901 and rottlerin (100 μM), REV 5901 and U73122 (100 μM) or REV 5901 and EGTA (2mM) supplemented DMEM and subjected to a single mechanical impact. Samples were fixed with 4 % paraformaldehyde and stained with Alexa Fluor 488 phalloidin (5 μl/ml). Cells were visualised using CLSM prior to and at 2 h, 24 h and 48 h post mechanical impact. Linear profiling analysis was used to quantify f-actin, expressed relative to volume changes. Treatment with rottlerin and U73122 was observed to significantly increase the initial rate of mechanical impact induced f-actin decrease compared to REV 5901. Additionally, treatment with rottlerin and U73122 was observed to have no effect on the initial REV 5901 induced increase in f-actin observed prior to mechanical impact. N=12 and n=60 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005 all vs. control. Arrow denotes impact point.*

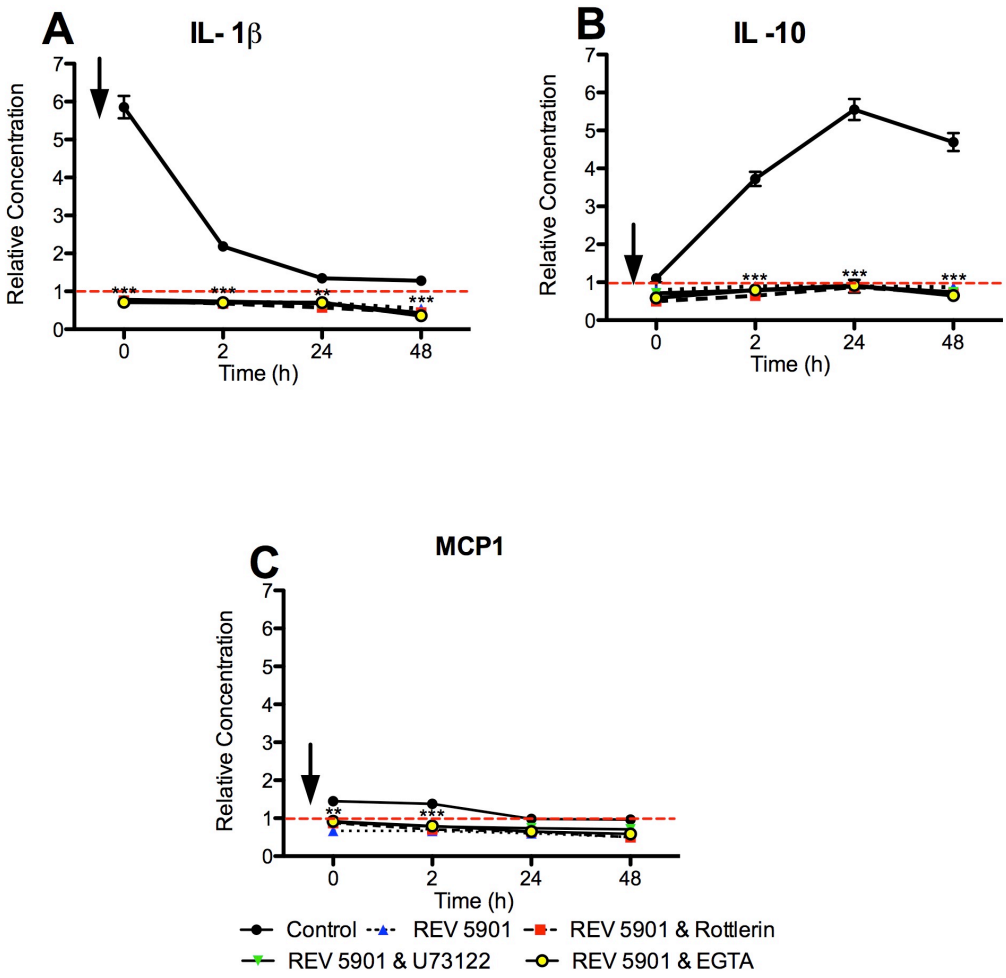


Figure 4.12: REV 5901 decreases inflammatory cytokines post-mechanical impact independent of PKC/PLCβ_3.

*Cartilage explants were pre-treated for 1 h with control isotonic, REV 5901 (50 μM), REV 5901 and rottlerin (100 μM), REV 5901 and U73122 (100 μM) or REV 5901 and EGTA (2mM) supplemented DMEM and subjected to a single mechanical impact. Supernatant samples immediately following and at 2 h, 24 h and 48 h post mechanical impact were analysed by ELISA and cytokine concentration expressed relative to non-impacted control samples. Treatment with rottlerin, U73122 and EGTA was observed to have no effect on the decrease in IL-1β (A), IL-10 (B) or MCP-1 (C) concentration observed with REV 5901. N=12 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005. Arrow denotes impact point.*

4.2.3 Tamoxifen as a Possible Chondroprotective Drug

Tamoxifen is known to be an inhibitor of RVD (Hall, 1995), in addition to having anticalmodulin actions. Thus, as calmodulin is an upstream molecule of actin depolymerising protein cofilin (Ono, 2007) and IIAD has been previously shown to be a feature of mechanical impact, tamoxifen has the potential for chondroprotective actions. Initially, it was necessary to confirm any chondroprotective effects induced by tamoxifen were not the result of osmotic changes. Thus, the osmolarity of Tamoxifen supplemented DMEM was measured as previously described (*See Materials and Methods*), where it was verified that Tamoxifen (286.01±8.33 mOsm) did not significantly adjust medium osmolarity compared to control (280.10±0.75 mOsm, *Fig. 4.13*). Therefore, the effects of Tamoxifen on chondrocyte viability and volume post mechanical impact were investigated, and compared to the previously noted chondroprotective effects of hyperosmotic challenge.

Tamoxifen was observed to have chondroprotective effects post mechanical impact, such that a viability of 88.30±3.34 % was noted at 48 h, not significantly different to viability prior to impact (95.02±4.51 %). Furthermore, the significant ($p<0.005$) reduction in initial cell death observed (0.50±0.03 $\%.h^{-1}$) was similar to the chondroprotective decrease in initial cell death observed with hyperosmotic challenge (0.50±0.04 $\%.h^{-1}$; *Fig. 4.14*).

Investigation into cell volume revealed Tamoxifen induced a decrease in cell volume to 423.99±15.54 μm^3 (*Fig. 4.15C*), compared to both control (716.14±37.81 μm^3; *Fig. 4.15A*) and hyperosmotic challenge (578.46±25.49 μm^3; *Fig. 4.15B*) prior to mechanical impact (*Fig. 4.15D*). Additionally, whilst the chondroprotective hyperosmotic challenge exhibited a biphasic IIVD, Tamoxifen significantly ($p<0.005$) decreased both the initial and subsequent rates of IIVD, to 1.19±0.30 $\mu m^3.h^{-1}$ and 0.30±0.14 $\mu m^3.h^{-1}$ respectively. The reduction of IIVD resulted in a cell volume of 432.29±5.45 μm^3 by 48 h post mechanical impact, not significantly different to cell volume prior to impact (*Fig. 4.16*).

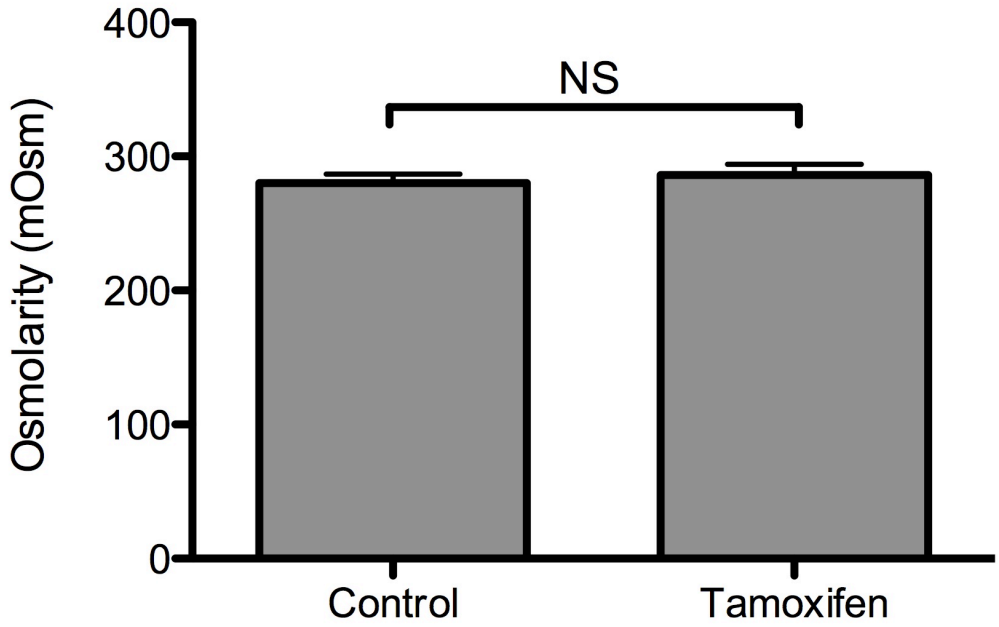
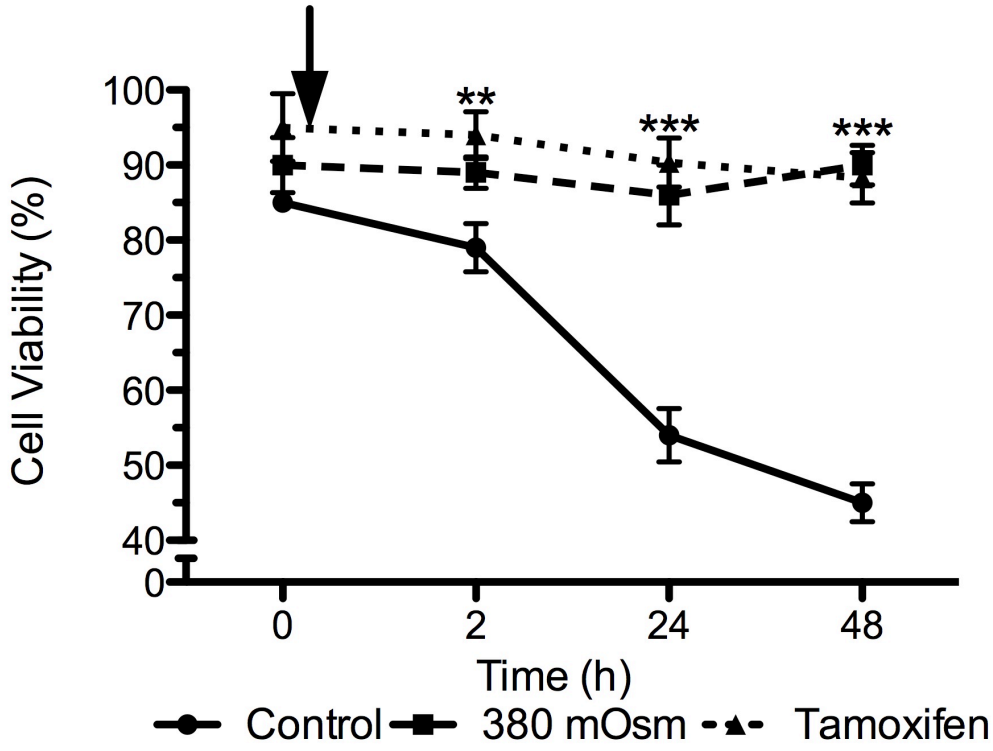


Figure 4.13: The addition of tamoxifen has no effect on medium osmolarity.
Samples of control isotonic and tamoxifen (10 μM) supplemented DMEM were measured for osmolarity using Vapro ™ vapour pressure osmometer. Tamoxifen was not observed to significantly alter medium osmolarity. N=25



Condition	Initial rate of chondrocyte death (%.h^{-1})	Subsequent rate of chondrocyte death (%.h^{-1})
Control	2.75 ± 0.14	0.66 ± 0.03
380 mOsm	0.50 ± 0.04 ***	0.00 ± 0.06 ***
Tamoxifen	0.50 ± 0.03 ***	0.12 ± 0.02 ***

Figure 4.14: Tamoxifen pre-treatment protects against cell death post mechanical impact.

*Cartilage explants were pre-treated for 1 h with control isotonic, hyperosmotic (380 mOsm) or tamoxifen (10 μM) supplemented DMEM and subjected to a single mechanical impact. Samples were loaded with Calcein AM (5 μM) and PI (1 μM) and visualised using CLSM prior to and at 2 h, 24 h and 48 h post mechanical impact. Imaris spot analysis was used to quantify cell viability. Tamoxifen was seen to protect against cell death. N=12 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005 all vs. control. Arrow denotes impact point.*

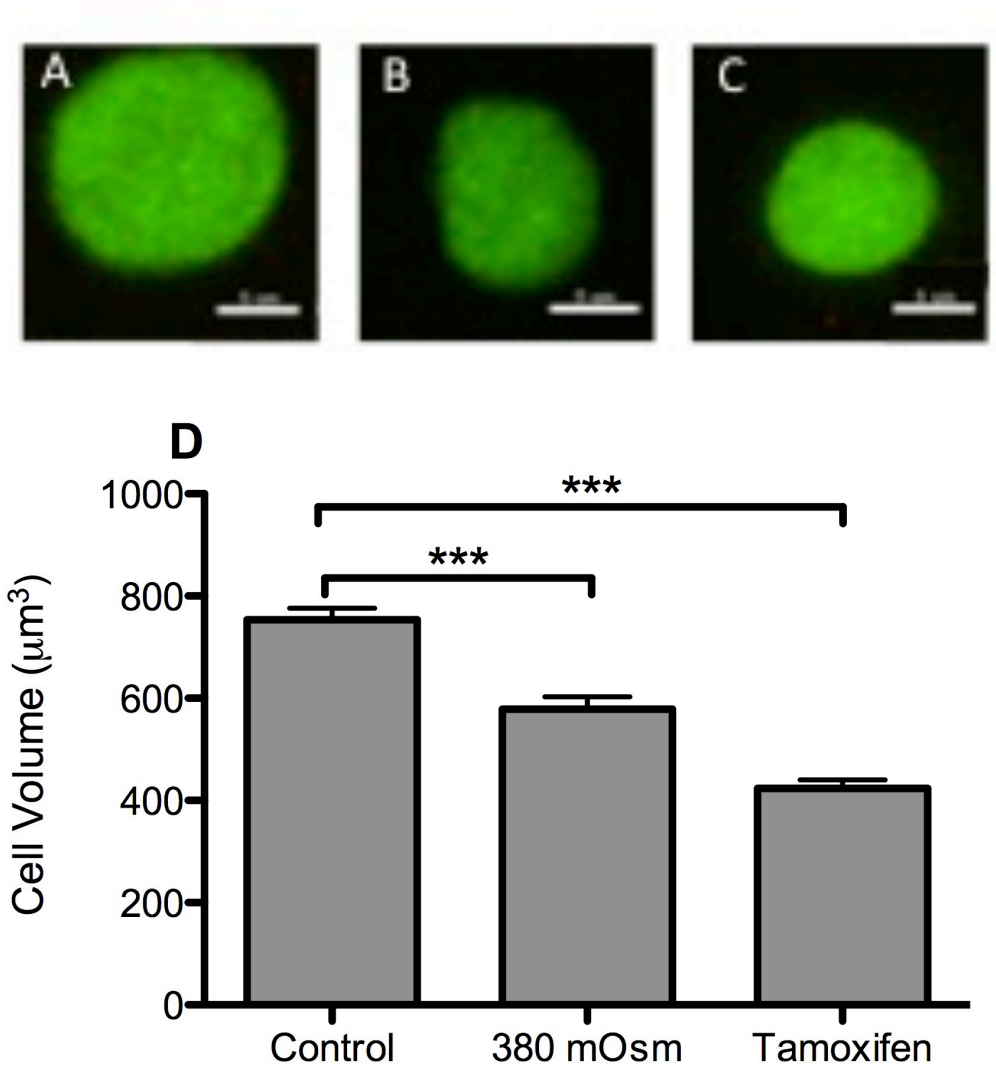
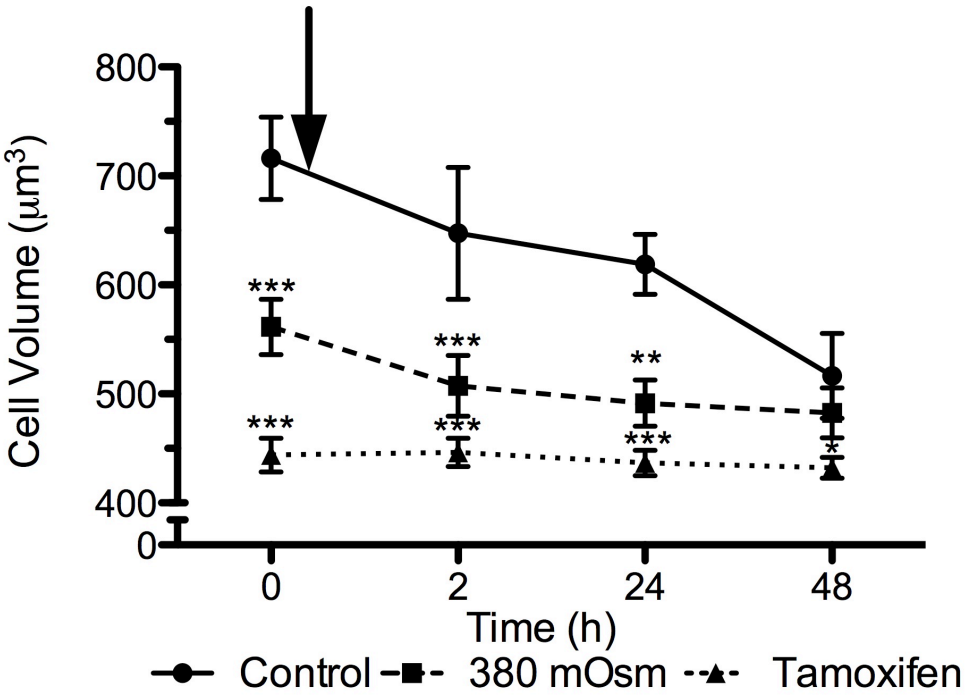


Figure 4.15: Tamoxifen decreases chondrocyte volume.

*Cartilage explants were pre-treated for 1 h with control isotonic (A), hyperosmotic (B; 380 mOsm) or tamoxifen (C; 10 μM) supplemented DMEM. Samples were loaded with Calcein AM (5 μM) and visualised using CLSM prior to mechanical impact. Isosurface volume analysis was used to quantify cell volume. Tamoxifen was observed to decrease cell volume prior to mechanical impact (D). N=12 and n=60 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005 all vs. control.*



Condition	Initial rate of chondrocyte volume decrease ($\mu m^3.h^1$)	Subsequent rate of chondrocyte volume decrease ($\mu m^3.h^{-1}$)
Control	34.38 ± 0.03	2.87 ± 0.85
380 mOsm	26.95±1.12 ***	0.54±0.05 ***
Tamoxifen	1.19 ± 0.30 ***	0.30 ± 0.14 ***

Figure 4.16: Tamoxifen protects against mechanical impact induced cell volume decrease.

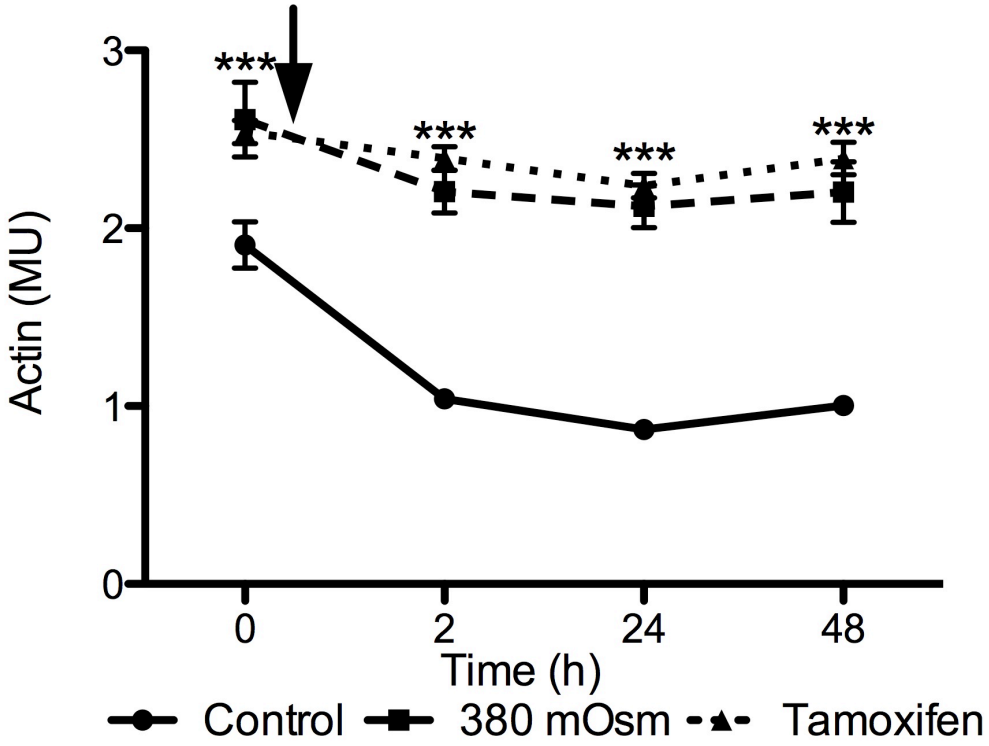
*Cartilage explants were pre-treated for 1 h with control isotonic, hyperosmotic (380 mOsm) or tamoxifen (10 μM) supplemented DMEM and subjected to a single mechanical impact. Samples were loaded with Calcein AM (5 μM) and visualised using CLSM prior to and at 2 h, 24 h and 48 h post mechanical impact. Isosurface volume analysis was used to quantify cell volume. Tamoxifen was observed to protect against mechanical impact induced cell volume decrease. N=12 and n=60 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005 all vs. control. Arrow denotes impact point.*

The effects of tamoxifen on the responses of the F-actin cytoskeleton to mechanical impact were investigated, to determine the role of the potential modulation of actin depolymerisation by tamoxifen within the chondroprotective mechanism. Thus, articular cartilage samples were fixed with 4% paraformaldehyde and stained for F-actin with Alexa Fluor 488 Phalloidin (5 μl/ml) prior to and 2, 24 and 48 h post mechanical impact. F-actin was imaged using CLSM and analysed using linear profiling and the PQ Factor, as previously described (*See Methods and Materials*).

Tamoxifen was observed to induce an initial increase in F-actin by 33 % to 2.54±0.07 MU, comparable to the increase induced by hyperosmotic challenge to 2.61±0.21 MU (*Fig. 4.17*). Additionally, tamoxifen protected against IIAD, significantly decreasing the rate of IIAD to 0.07±0.00 $MU.h^{-1}$, a greater decrease than that exhibited by hyperosmotic challenge (*Fig. 4.17*).

The chondroprotective mechanism of tamoxifen was further investigated to determine any immunomodulatory component, whereby supernatant samples were collected immediately and at 2, 24 and 48 h post mechanical impact and analysed by ELISA for cytokine concentration as previously described (*See Methods and Materials*).

Tamoxifen was noted to have similar immunomodulatory effects as hyperosmotic challenge; the initial peak in IL-1β concentration observed under control conditions was decreased by 58 % (*Fig. 4.18A*) prior to declining to non-impact baseline levels by 24 h post impact. Similarly, Tamoxifen induced a decrease in peak IL-10 concentration (*Fig. 4.18B*) at 24 h by 22 %, but did not change the cytokine expression profile induced by mechanical impact. MCP-1 concentration was not noted to be significantly different at any time point post impact between tamoxifen, hyperosmotic or control samples (*Fig. 4.18C*).



Condition	Initial rate of actin polymerisation ($MU.h^{-1}$)	Subsequent rate of actin polymerisation ($MU.h^{-1}$)
Control	-0.43 ± 0.03	0.00 ± 0.00
380 mOsm	-0.20 ± 0.04 ***	0.01 ± 0.00
Tamoxifen	-0.07 ± 0.00 ***	0.00 ± 0.00

Figure 4.17: Tamoxifen protects against mechanical impact induced F-actin decrease.

*Cartilage explants were pre-treated for 1 h with control isotonic, hyperosmotic (380 mOsm) or tamoxifen (10 μM) supplemented DMEM and subjected to a single mechanical impact. Samples were fixed with 4 % paraformaldehyde and stained with Alexa Fluor 488 phalloidin (5 μl/ml). Cells were visualised using CLSM prior to and at 2 h, 24 h and 48 h post mechanical impact. Linear profiling analysis was used to quantify f-actin, expressed relative to volume changes. Tamoxifen was observed to increase f-actin prior to mechanical impact, and additionally, to protect against mechanical impact induced f-actin decrease. N=12 and n=60 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005 all vs. control. Arrow denotes impact point.*

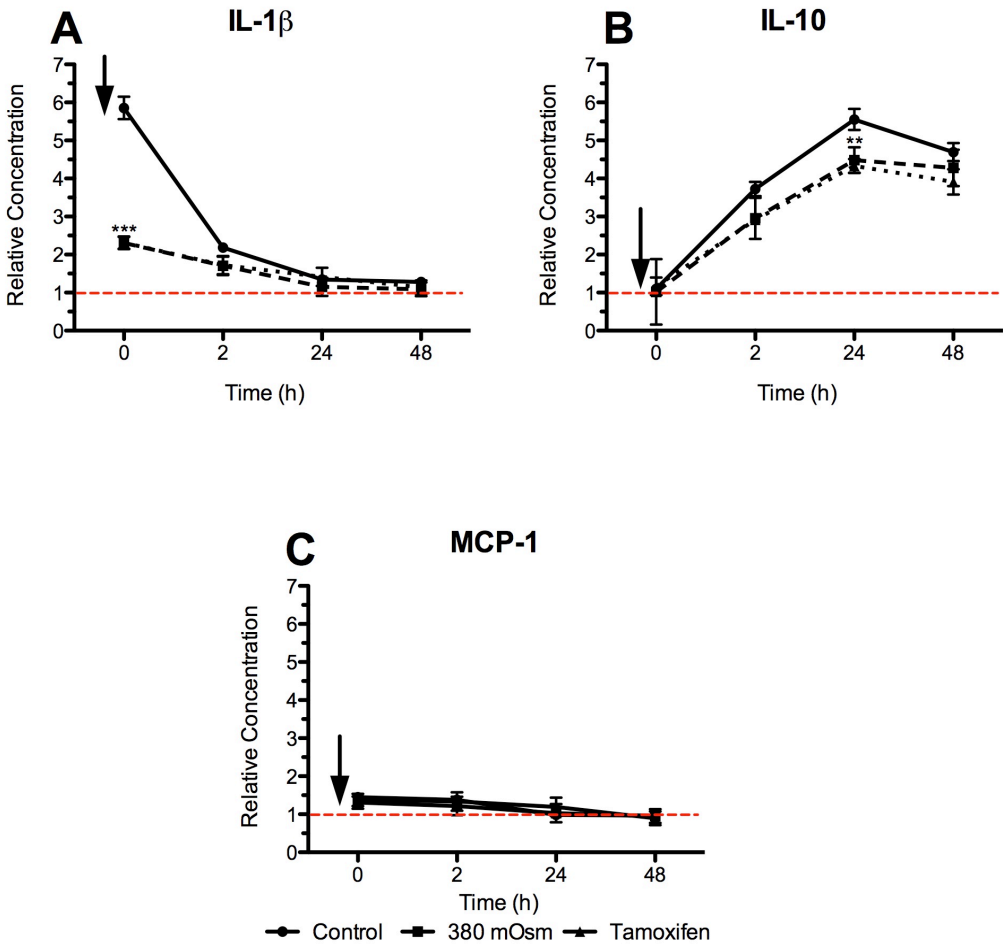


Figure 4.18: Tamoxifen decreases inflammatory cytokines post-mechanical impact. *Cartilage explants were pre-treated for 1 h with control isotonic, hyperosmotic (380 mOsm) or tamoxifen (10 μM) supplemented DMEM and subjected to a single mechanical insult. Supernatant samples immediately following and at 2 h, 24 h and 48 h post mechanical impact were analysed by ELISA and cytokine concentration expressed relative to non-impacted control samples. Tamoxifen was observed to decrease both pro-inflammatory (IL-1β; A and MCP-1; C) and anti-inflammatory (IL-10; B) cytokines post mechanical impact when compared to control. N=12 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005. Arrow denotes impact point.*

4.2.4 Nutritional supplements: Chondroprotective Effects

Nutritional supplements, or nutriceuticals, are used by the general public to maintain 'healthy joints', and two of the most commonly used are chondroitin sulphate and glucosamine sulphate (ARC, 2009). Chondroitin sulphate has been reported to stimulate cartilage repair and have anti-inflammatory actions (Huskisson, 2008, Deal and Moskowitz, 1999) and glucosamine sulphate is the principal substrate in the synthesis of PGs (Simanek *et al*., 2005). To investigate any potential chondroprotective or immunomodulatory effects of these nutriceuticals, the osmolarity of chondroitin and glucosamine sulphate supplemented DMEM was first analysed to rule out the possibility of the nutriceuticals acting via osmotic mechanisms. It was observed that chondroitin sulphate (282.20±2.48 mOsm) and glucosamine sulphate (280.21±4.02 mOsm) did not significantly adjust medium osmolarity compared to control (280.10±0.75 mOsm, *Fig. 4.19*). Subsequently, articular cartilage explants were pre-incubated with chondroitin sulphate supplemented DMEM of 0.1, 0.2 or 0.3 mg/ml, or glucosamine sulphate supplemented DMEM of 0.025, 0.05 or 0.1 mg/ml prior to mechanical impact, in order to determine the optimal concentration for further investigation.

A decrease in cell viability was observed post-mechanical impact under glucosamine sulphate conditions up to 0.025 mg/ml. However, glucosamine sulphate of 0.05 and 0.1 mg/ml exhibited chondroprotective actions, with no significant cell death by 48 h post mechanical impact (0.05 mg/ml: 80.12±4.45 %, 0.1 mg/ml: 90.10±3.25 %; *Fig. 4.20A*). Similarly, chondroitin sulphate was observed to be chondroprotective at concentrations of 0.2 mg/ml and above, with no significant cell death at 48 h post mechanical impact (0.2 mg/ml: 84.29±1.20 %, 0.3 mg/ml: 90.06±2.12 %; *Fig. 4.20B*).

From these data it was concluded 0.1 mg/ml glucosamine sulphate and 0.3 mg/ml chondroitin sulphate had the most potential chondroprotective effects, and were used henceforth.

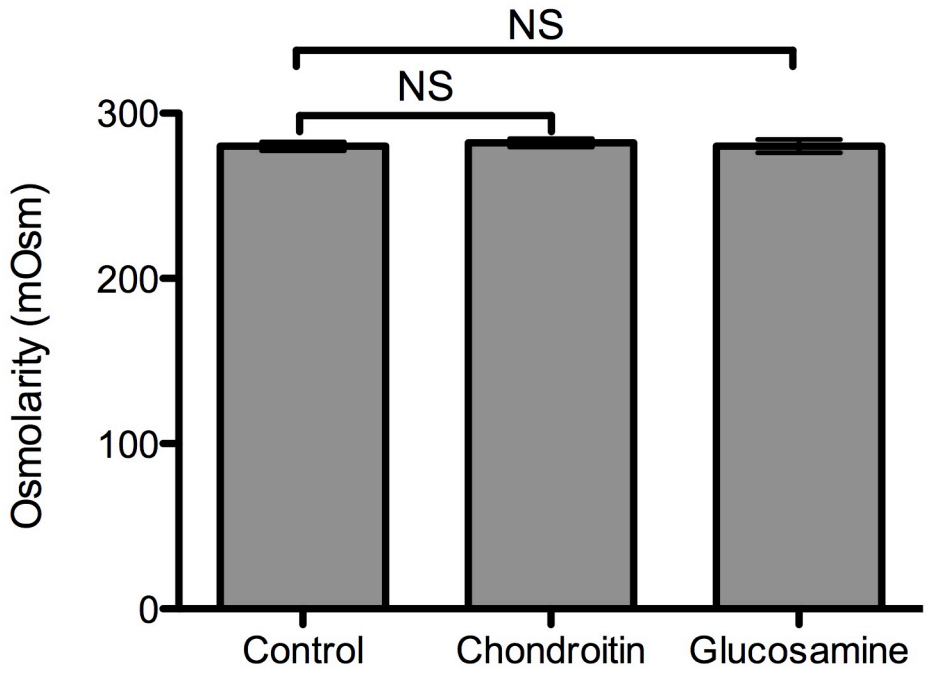


Figure 4.19: The addition of chondroitin sulphate or glucosamine sulphate had no effect on medium osmolarity.

Samples of control isotonic, chondroitin sulphate (0.3 mg/ml) and glucosamine sulphate (0.1 mg/ml) supplemented DMEM were measured for osmolarity using Vapro ™ vapour pressure osmometer. Neither chondroitin or glucosamine sulphate was observed to significantly alter medium osmolarity. N=25

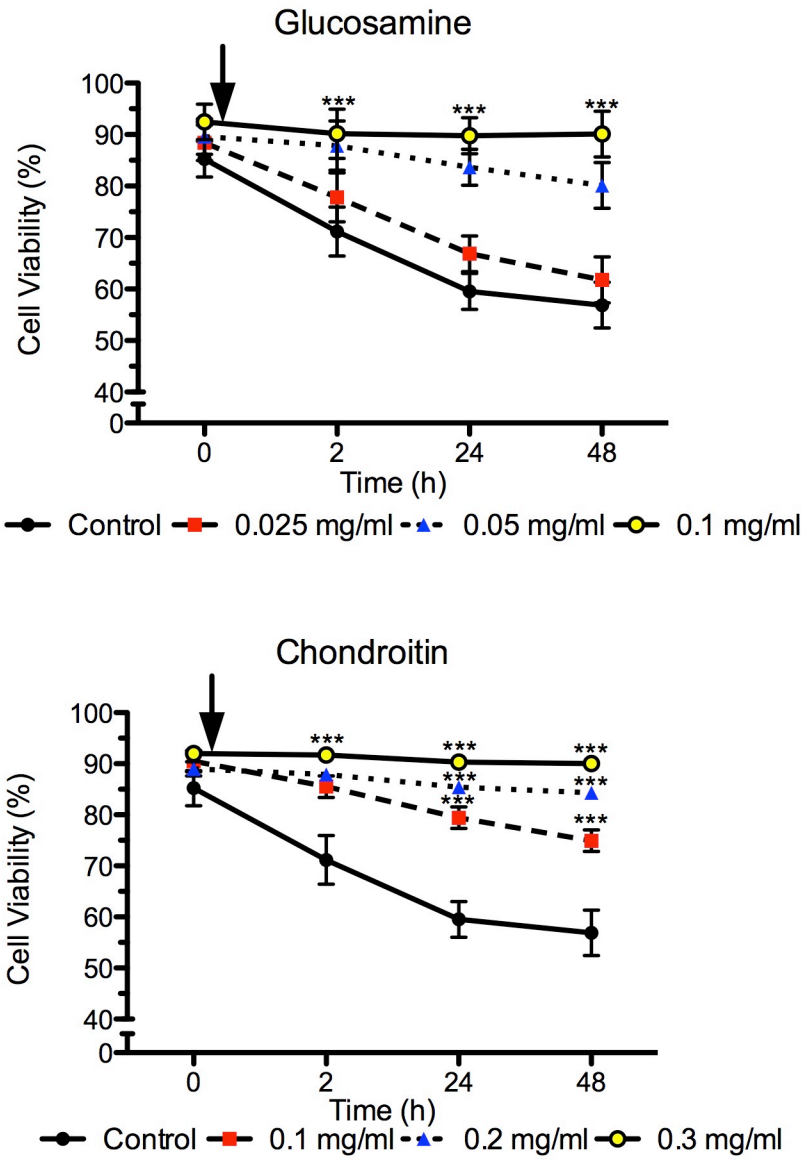


Figure 4.20: Chondroitin sulphate and glucosamine sulphate pre-treatment decreases chondrocyte death post-mechanical impact.

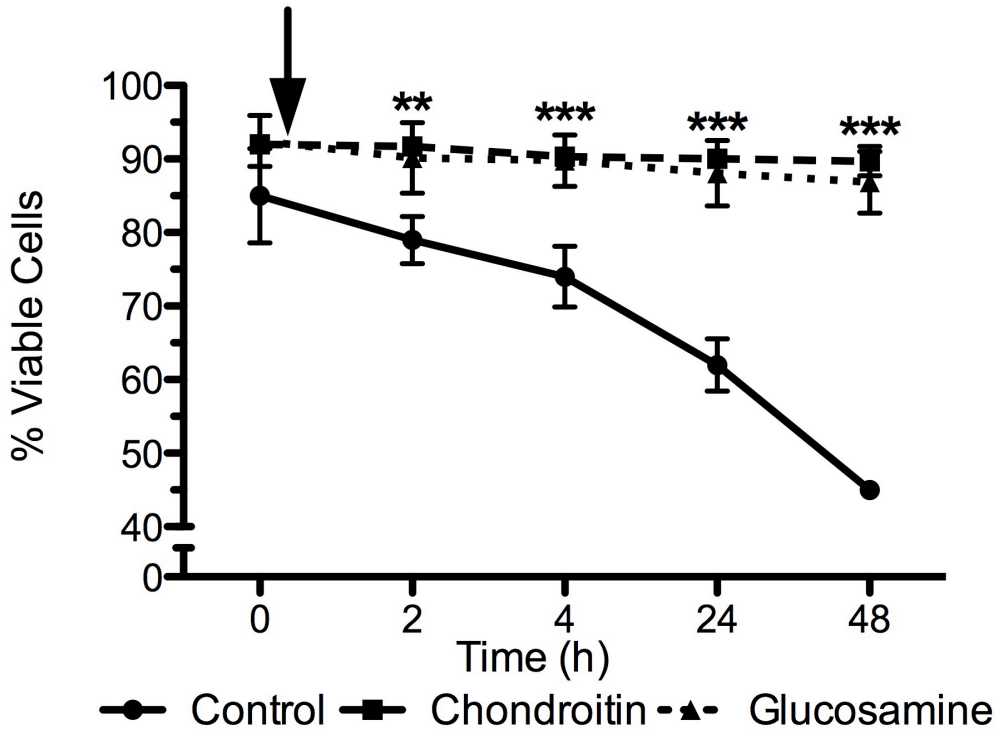
*Cartilage explants were pre-treated for 1hr with control isotonic, glucosamine sulphate supplemented DMEM of 0.025, 0.05 or 0.1 mg/ml (A) or chondroitin sulphate supplemented DMEM of 0.1, 0.2 or 0.3 mg/ml (B) and subjected to a single mechanical impact. Samples were loaded with Calcein AM (5 μM) and PI (1 μM) and visualised using CLSM prior to and at 2 h, 4 h, 24 h and 48 h post mechanical impact. Imaris Spot analysis was used to quantify the number of viable and non-viable cells. Glucosamine sulphate was observed to increase cell viability at concentrations of 0.05 mg/ml and above. Chondroitin sulphate was observed to increase cell viability at all concentrations. N=12 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005. Arrow denotes impact point.*

It was observed that chondroitin sulphate significantly decreased the initial rate of cell death post mechanical impact to 0.42±0.17 %.h^{-1}, compared to control rate of 2.75±0.14 %.h^{-1}, and additionally decreased the subsequent rate of cell death to 0.01±0.01 %.h^{-1}, thus exhibiting chondroprotective effects. Similarly, glucosamine sulphate treatment decreased the initial rate of cell death to 0.67±0.59 %.h^{-1}, and the subsequent cell death to 0.06±0.02 %.h^{-1} (*Fig. 4.21*). Chondroitin and glucosamine sulphate were therefore confirmed to have chondroprotective effects.

As cell shrinkage was previously confirmed to be a chondroprotective mechanism, the effects of chondroitin and glucosamine sulphate on chondrocyte volume were investigated. Both chondroitin (*Fig. 4.22B*) and glucosamine sulphate (*Fig. 4.22C*) were noted to significantly decrease cell volume prior to mechanical impact by 37 % and 27 % respectively (*Fig. 4.22D*). Additionally, whilst neither chondroitin or glucosamine sulphate exhibited an effect on the initial rate of IIVD, both decreased the subsequent rate of IIVD from 2.87±0.85 $\mu m^3.h^{-1}$ to 0.71±0.18 $\mu m^3.h^{-1}$ and 1.07±0.05 $\mu m^3.h^{-1}$ respectively (*Fig. 4.23*).

Furthermore, the effects of nutriceuticals on the F-actin response to mechanical impact were determined to further investigate the mechanism of chondroprotection, whereby articular cartilage samples were fixed with 4% paraformaldehyde and stained for F-actin with Alexa Fluor 488 Phalloidin (5 μl/ml) prior to and 2, 24 and 48 h post mechanical impact. F-actin was imaged using CLSM and analysed using linear profiling and the PQ Factor, as previously described (*See Methods and Materials*).

F-actin concentration prior to mechanical impact was significantly increased ($p<0.005$) upon treatment with chondroitin sulphate to 1.92±0.16 MU and with glucosamine sulphate to 2.14±0.18 MU, increases of 32 % and 27 % respectively (*Fig. 4.24*). Additionally, both chondroitin and glucosamine sulphate exhibited significant ($p<0.005$) protection against IIAD, decreasing the rate of IIAD from 0.43±0.03 MU.h^{-1}, under control conditions, to 0.08±0.04 MU.h^{-1} and 0.11±0.04 MU.h^{-1} correspondingly (*Fig. 4.24*).



Condition	Initial rate of chondrocyte death (%.h^{-1})	Subsequent rate of chondrocyte death (%.h^{-1})
Control	2.75 ± 0.14	0.66 ± 0.03
Chondroitin	0.42 ± 0.17 ***	0.01 ± 0.01 ***
Glucosamine	0.67 ± 0.59 ***	0.06 ± 0.02 ***

Figure 4.21: Chondroitin sulphate and glucosamine sulphate protected against cell death post mechanical impact.

*Cartilage explants were pre-treated for 1 h with control isotonic, chondroitin sulphate (0.3 mg/ml) or glucosamine sulphate (0.1 mg/ml) supplemented DMEM and subjected to a single mechanical impact. Samples were loaded with Calcein AM (5 μM) and PI (1 μM) and visualised using CLSM prior to and at 2 h, 4 h, 24 h and 48 h post mechanical impact. Imaris spot analysis was used to quantify cell viability. Chondroitin sulphate and glucosamine sulphate were seen to decrease both the initial and subsequent rate of cell death. N=12 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005 all vs. control. Arrow denotes impact point.*

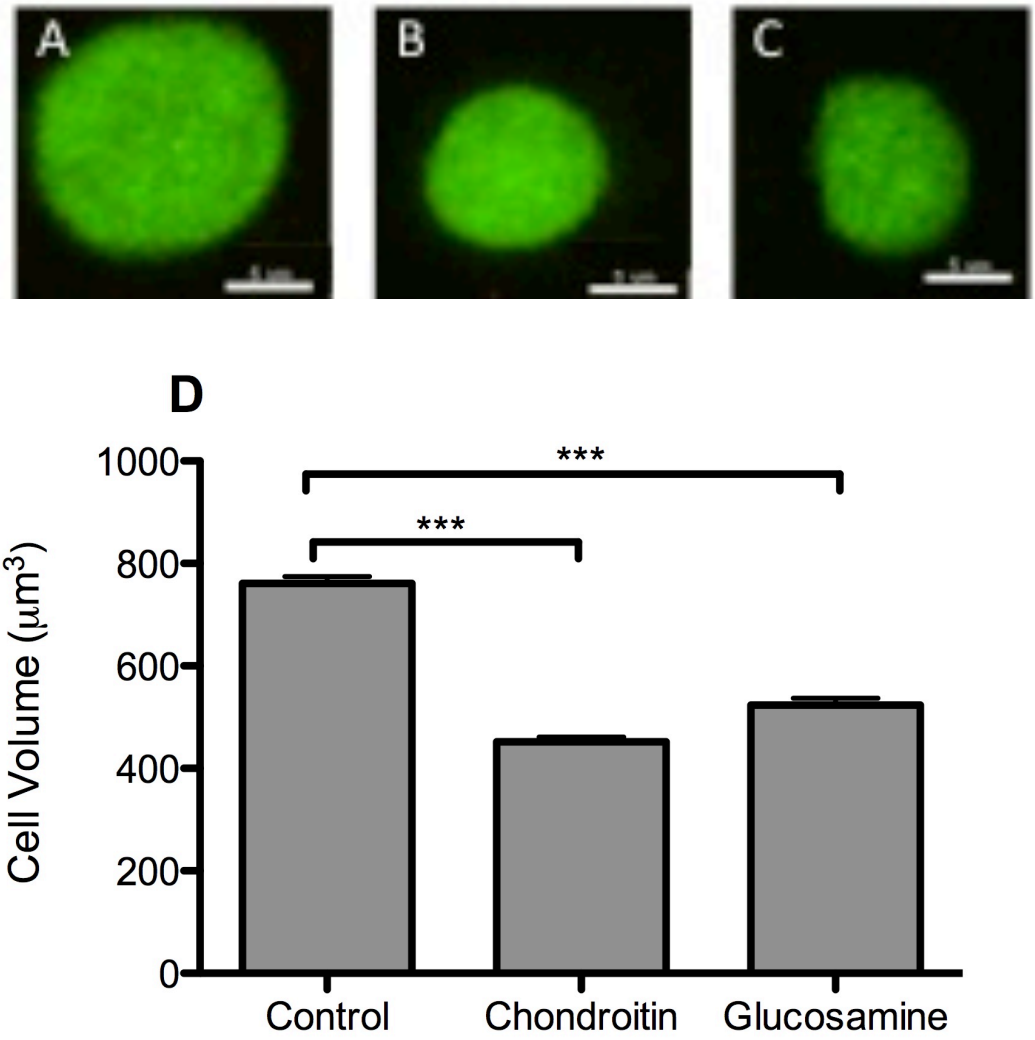
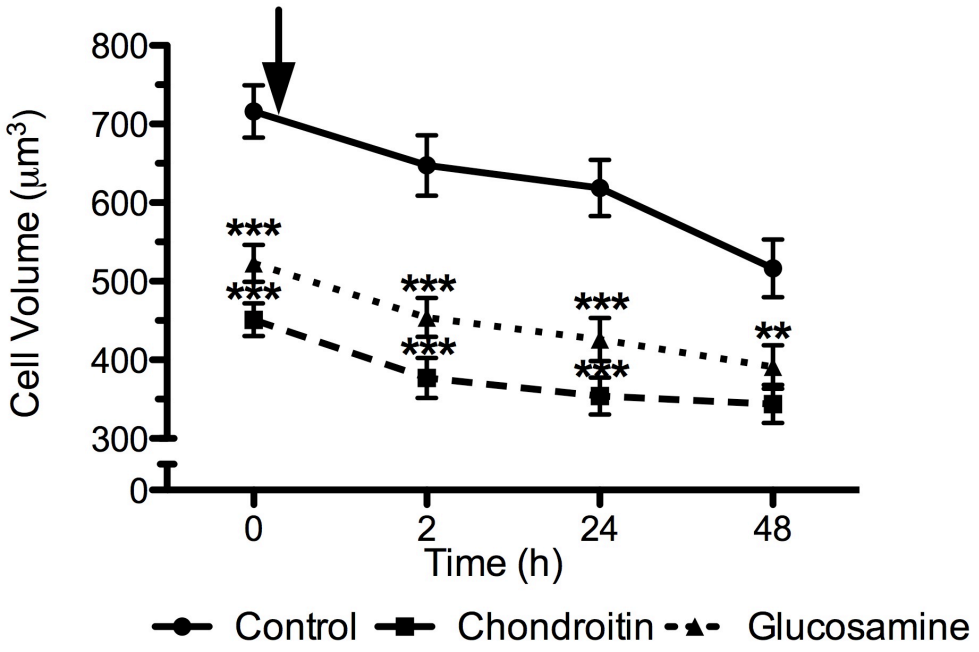


Figure 4.22: Chondroitin sulphate and glucosamine sulphate decrease chondrocyte volume.

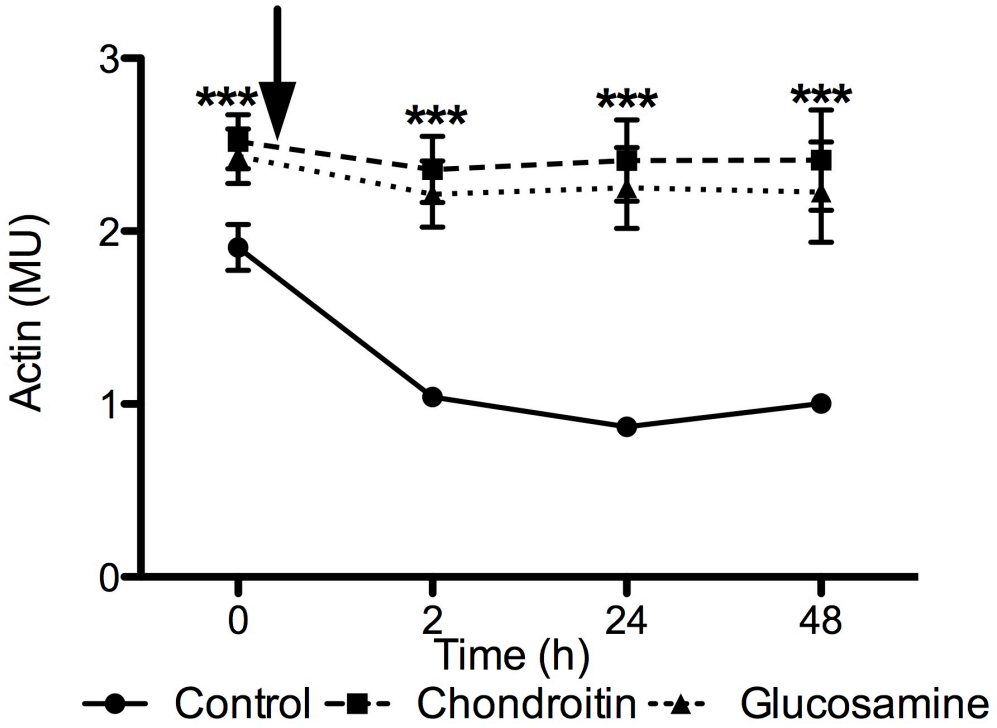
*Cartilage explants were pre-treated for 1 h with control isotonic (A), chondroitin sulphate (0.3 mg/ml; B) or glucosamine sulphate (0.1 mg/ml; C) supplemented DMEM. Samples were loaded with Calcein AM (5 μM) and visualised using CLSM prior to mechanical impact. Isosurface volume analysis was used to quantify cell volume. Both chondroitin and glucosamine sulphate were observed to decrease cell prior to mechanical impact (D). N=12 and n=60 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005 all vs. control.*



Condition	Initial rate of chondrocyte volume decrease ($\mu m^3.h^{-1}$)	Subsequent rate of chondrocyte volume decrease ($\mu m^3.h^{-1}$)
Control	34.38 ± 0.03	2.87 ± 0.85
Chondroitin	37.00 ± 0.04	0.71 ± 0.18 ***
Glucosamine	34.50 ± 0.04	1.07 ± 0.05 **

Figure 4.23: Chondroitin sulphate and glucosamine sulphate protect against mechanical impact induced decrease in cell volume.

*Cartilage explants were pre-treated for 1 h with control isotonic, chondroitin sulphate (0.3 mg/ml) or glucosamine sulphate (0.1 mg/ml) supplemented DMEM and subjected to a single mechanical impact. Samples were loaded with Calcein AM (5 μM) and visualised using CLSM prior to and at 2 h, 24 h and 48 h post mechanical impact. Isosurface volume analysis was used to quantify cell volume. Both chondroitin and glucosamine sulphate were observed to significantly reduce the subsequent rate of mechanical impact induced cell volume decrease. N=12 and n=60 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005 all vs. control. Arrow denotes impact point.*



Condition	Initial rate of actin polymerisation ($MU.h^{-1}$)	Subsequent rate of actin polymerisation ($MU.h^{-1}$)
Control	-0.43 ± 0.03	0.00 ± 0.00
Chondroitin	-0.08 ± 0.04 ***	0.01 ± 0.01
Glucosamine	-0.11 ± 0.04 ***	0.00 ± 0.00

Figure 4.24: Chondroitin sulphate and glucosamine sulphate protect against mechanical impact induced decrease in F-actin.

*Cartilage explants were pre-treated for 1 h with control isotonic, chondroitin sulphate (0.3 mg/ml) or glucosamine sulphate (0.1 mg/ml) supplemented DMEM and subjected to a single mechanical impact. Samples were fixed with 4 % paraformaldehyde and stained with Alexa Fluor 488 phalloidin (5 μl/ml). Cells were visualised using CLSM prior to and at 2 h, 24 h and 48 h post mechanical impact. Linear profiling analysis was used to quantify f-actin, expressed relative to volume changes. Both chondroitin and glucosamine sulphate were observed to increase f-actin prior to mechanical impact and subsequently protect against impact induced actin decrease. N=12 and n=60 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005 all vs. control. Arrow denotes impact point.*

The role of possible immunomodulatory effects in the chondroprotective mechanism of chondroitin and glucosamine sulphate was investigated, whereby supernatant samples were collected immediately and at 2, 24 and 48 h post mechanical impact and analysed by ELISA for cytokine concentration as previously described (*See Methods and Materials*).

Chondroitin and glucosamine sulphate were observed to inhibit the cytokine response to mechanical impact. Peak IL-1β concentration, observed at 0 h post mechanical impact under control conditions, was decreased from 5.86±0.29 AU to 1.03±0.06 AU and 1.05±0.07 AU respectively, concentrations not significantly different from non-impact (non-impact concentrations = 1 AU). Furthermore, IL-1β was maintained at non-impact levels for 0-48 h post mechanical impact by both chondroitin and glucosamine sulphate (*Fig. 4.25A*). A similar effect was observed on IL-10 release (*Fig. 4.25B*), whereby chondroitin and glucosamine sulphate prevented the increase in IL-10 observed in response to mechanical impact under control conditions, again maintaining cytokine concentration at non-impact levels for the 48 h post mechanical impact. Furthermore, MCP-1 release (*Fig. 4.25C*) was decreased to near non-impact levels by glucosamine sulphate (0.98±0.03 AU) and maintained for the 48 h post impact. Interestingly, chondroitin sulphate decreased MCP-1 concentration to 0.58±0.03 AU immediately post impact, below non-impact levels. MCP-1 release further decreased between 0-48 h, reaching a low of 0.40±0.04 AU at 48 h post impact (*Fig. 4.25C*).

Chondroitin and glucosamine sulphate were therefore confirmed to have chondroprotective effects, associated with a decrease in initial chondrocyte volume, an increase in initial F-actin and prevention of cytokine release.

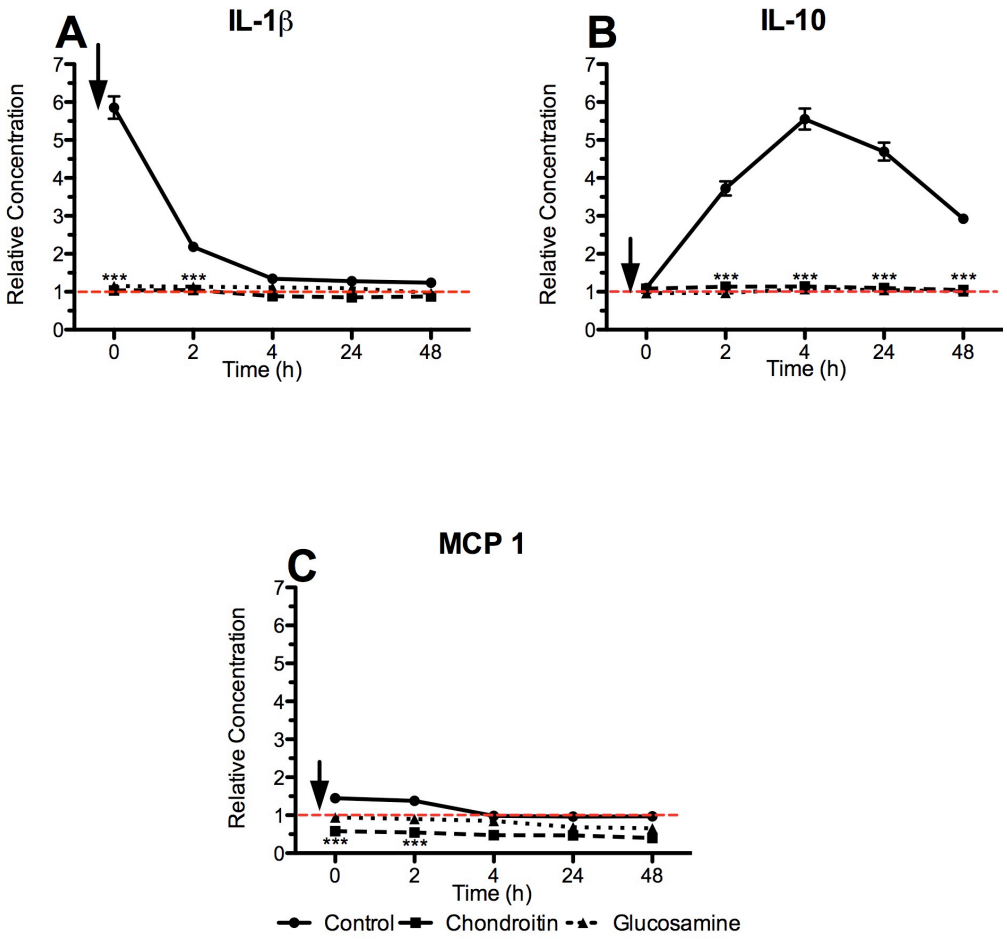


Figure 4.25: Chondroitin sulphate and glucosamine sulphate decrease inflammatory cytokines post-mechanical impact.

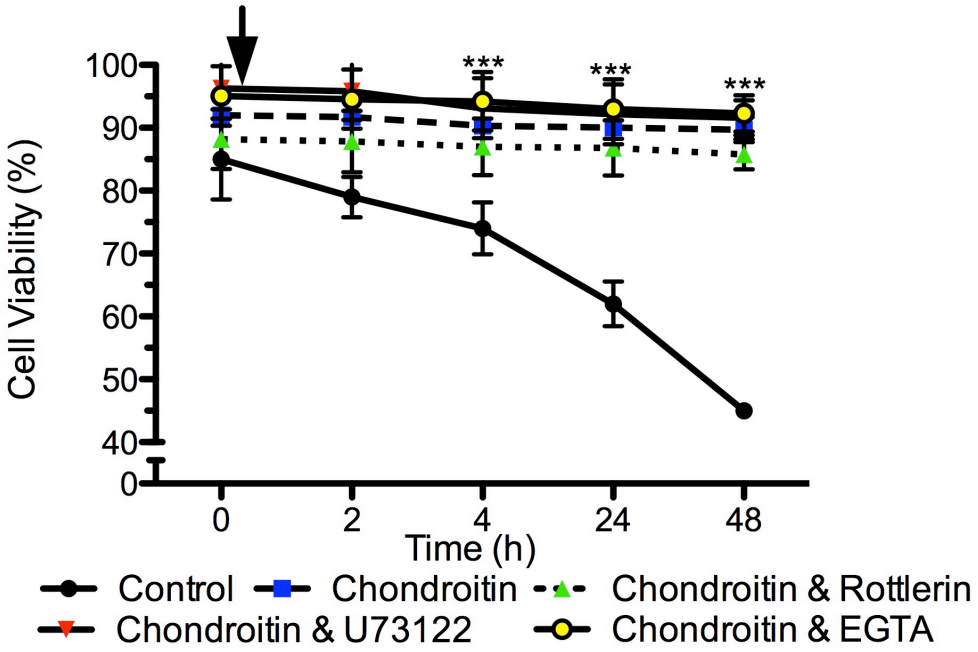
*Cartilage explants were pre-treated for 1 h with control isotonic, chondroitin sulphate (0.3 mg/ml) or glucosamine sulphate (0.1 mg/ml) supplemented DMEM and subjected to a single mechanical impact. Supernatant samples immediately following and at 2 h, 4h, 24 h and 48 h post mechanical impact were analysed by ELISA and cytokine concentration expressed relative to non-impacted control samples. Both chondroitin and glucosamine sulphate were observed to significantly decrease IL-1β (A), IL-10 (B) and MCP-1 (C) over the whole time course when compared to both control. N=12 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005. Arrow denotes impact point.*

4.2.5 The Role of Calcium Signalling in the Mechanism of Action of Nutritional Supplements

Having confirmed the role of intracellular calcium ($[Ca^{2+}]_i$) signalling in conveying the chondroprotective effects of REV 5901 using inhibitors of the PKC/PLCβ_3 signalling pathway, the effect of these inhibitors on a proposed chondroprotective $[Ca^{2+}]_i$ dependent pathway of chondroitin and glucosamine sulphate were investigated, whereby samples were pre-incubated rottlerin (100 μM), U73122 (100 μM) or EGTA (2mM) and subsequently with chondroitin sulphate (0.3 mg/ml) or glucosamine sulphate (0.1 mg/ml).

Rottlerin, U73122 and EGTA were observed to not inhibit the chondroprotective effects seen with chondroitin sulphate post mechanical impact (48 h: Chondroitin sulphate: 89.71±1.98 %, Rottlerin: 85.78±2.38 %, U73122: 90.58±2.78 %, EGTA: 92.31±2.89 %; *Fig. 4.26*). Similarly, the chondroprotective effects of glucosamine were not significantly affected by rottlerin, U73122 or EGTA (48 h: Glucosamine sulphate: 86.85±1.19 %, Rottlerin: 84.54±3.69 %, U73122: 84.93±3.67 %, EGTA: 91.56±2.89 %; *Fig. 4.27*). Thus suggesting chondroitin and glucosamine exhibit chondroprotective effects via a mechanism independent of the PKC/PLCβ_3/IP_3 $[Ca^{2+}]_i$ signalling pathway.

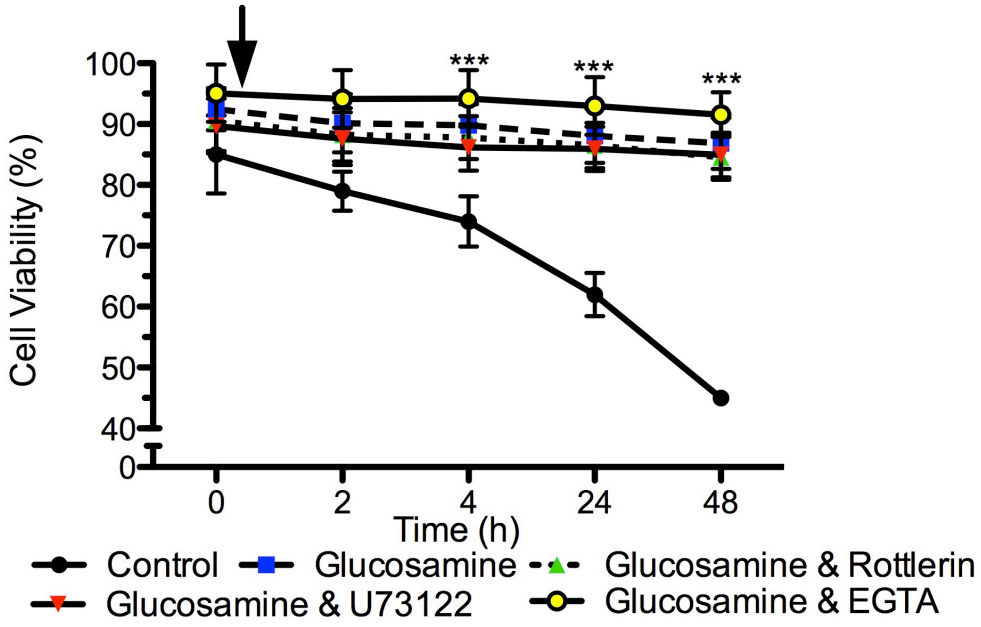
Interestingly, whilst rottlerin, U73122 and EGTA did not significantly effect the decrease in cell volume induced by chondroitin and glucosamine sulphate prior to mechanical impact, rottlerin and U73122 were observed to inhibit IIVD, seen with both chondroitin and glucosamine sulphate and control (*Fig. 4.28/29*), whereby the initial rate of IIVD was significantly ($p<0.005$) decreased to 4.91±0.04 $\mu m^3.h^{-1}$ and 13.70±0.30 $\mu m^3.h^{-1}$ by rottlerin, and 11.00±0.34 $\mu m^3.h^{-1}$ and 6.21±0.03 $\mu m^3.h^{-1}$ by U73122 for chondroitin sulphate and glucosamine sulphate samples respectively (*Fig. 4.28/29*). These data further supports the role of $[Ca^{2+}]_i$ signalling within the IIVD mechanism.



Condition	Initial rate of chondrocyte death (%.h^{-1})	Subsequent rate of chondrocyte death (%.h^{-1})
Control	2.75 ± 0.14	0.66 ± 0.03
Chondroitin	0.42 ± 0.17 ***	0.01 ± 0.01 ***
Chondroitin + Rottlerin	0.30 ± 0.05 ***	0.03 ± 0.01 ***
Chondroitin + U73122	0.59 ± 0.03 ***	0.03 ± 0.01 ***
Chondroitin + EGTA	0.21 ± 0.03 ***	0.04 ± 0.01 ***

Figure 4.26: Chondroitin sulphate's chondroprotective actions are not PKC/PLCβ_3 dependant.

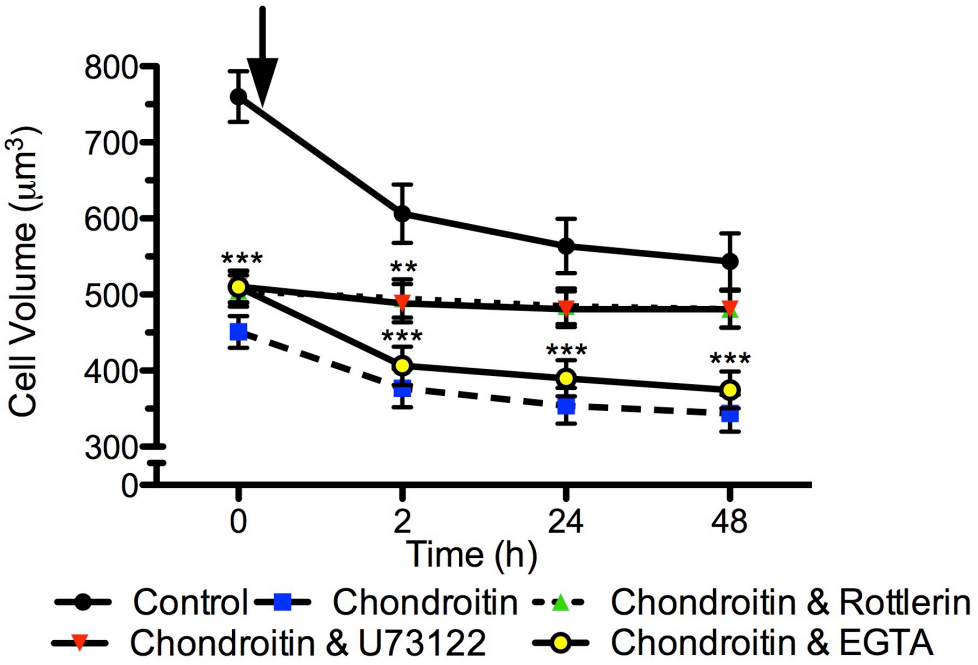
*Cartilage explants were pre-treated for 1 h with control isotonic, chondroitin sulphate (0.3 mg/ml) or chondroitin sulphate with rottlerin (100 μM), U73122 (100 μM) or EGTA (2mM) and subjected to a single mechanical impact. Samples were loaded with Calcein AM (5 μM) and PI (1 μM) and visualised using CLSM prior to and at 2 h, 4 h, 24 h and 48 h post mechanical impact. Imaris spot analysis was used to quantify cell viability. Treatment with rottlerin, U73122 and EGTA was observed to not significantly affect the chondroprotective effects of chondroitin sulphate. N=12 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005 all vs. control. Arrow denotes impact point.*



Condition	Initial rate of chondrocyte death (%.h^{-1})	Initial rate of chondrocyte death (%.h^{-1})
Control	2.75 ± 0.14	0.66 ± 0.03
Glucosamine	0.67 ± 0.59 ***	0.06 ± 0.02 ***
Glucosamine + Rottlerin	0.68 ± 0.04 ***	0.04 ± 0.09 ***
Glucosamine + U73122	0.08 ± 0.01 ***	0.03 ± 0.01 ***
Glucosamine + EGTA	0.21 ± 0.15 ***	0.06 ± 0.00 ***

Figure 4.27: Glucosamine sulphate's chondroprotective actions are not PKC/PLCβ_3 dependant.

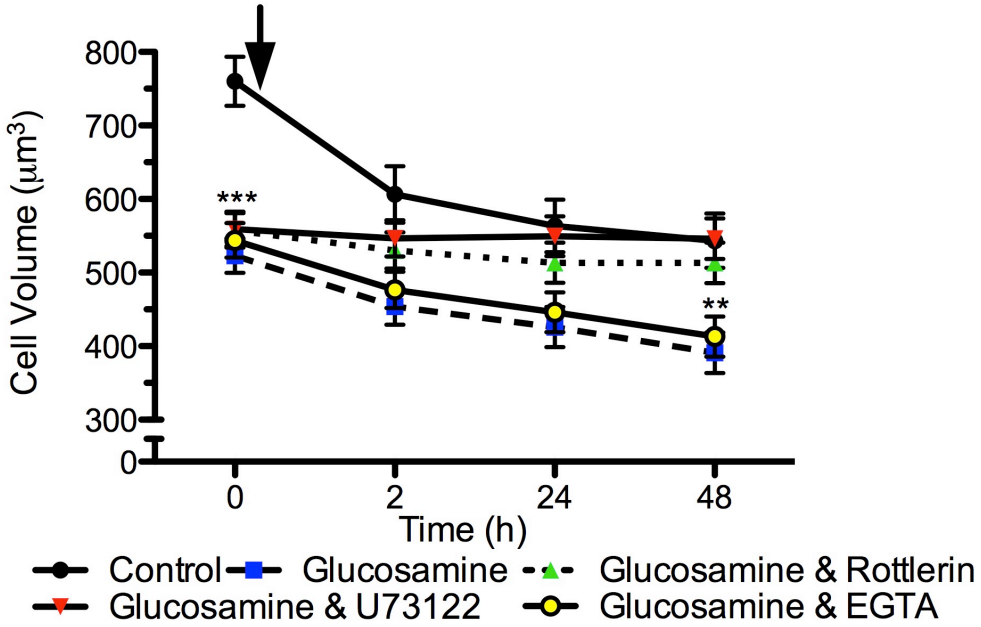
*Cartilage explants were pre-treated for 1 h with control isotonic, glucosamine sulphate (0.1 mg/ml) or glucosamine sulphate with rottlerin (100 μM), U73122 (100 μM) or EGTA (2mM) and subjected to a single mechanical impact. Samples were loaded with Calcein AM (5 μM) and PI (1 μM) and visualised using CLSM prior to and at 2 h, 4 h, 24 h and 48 h post mechanical impact. Imaris spot analysis was used to quantify cell viability. Treatment with rottlerin, U73122 and EGTA was observed to not significantly affect the chondroprotective effects of glucosamine sulphate. N=12 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005 all vs. control. Arrow denotes impact point.*



Condition	Initial rate of chondrocyte volume decrease ($\mu m^3.h^{-1}$)	Subsequent rate of chondrocyte volume decrease ($\mu m^3.h^{-1}$)
Control	34.38 ± 0.03	2.87 ± 0.85
Chondroitin	37.00 ± 0.04	0.71 ± 0.18 ***
Chondroitin + Rottlerin	4.91 ± 0.04 ***	0.31 ± 0.09 ***
Chondroitin + U73122	11.00 ± 0.34 ***	0.16 ± 0.01 ***
Chondroitin + EGTA	41.73 ± 0.35	0.69 ± 0.02 ***

Figure 4.28: Chondroitin sulphate decreases cell volume by PKC/PLCβ_3 independent mechanisms.

*Cartilage explants were pre-treated for 1 h with control isotonic, chondroitin sulphate (0.3 mg/ml) or chondroitin sulphate with rottlerin (100 μM), U73122 (100 μM) or EGTA (2mM) and subjected to a single mechanical impact. Samples were loaded with Calcein AM (5 μM) and visualised using CLSM prior to and at 2 h, 24 h and 48 h post mechanical impact. Isosurface volume analysis was used to quantify cell volume. Treatment with rottlerin, U73122 and EGTA was not observed to significantly affect initial cell volume compared to chondroitin sulphate controls, but rottlerin and U73122 inhibited the initial rate of impact induced volume decrease. N=12 and n=60 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005 all vs. control. Arrow denotes impact point.*



Condition	Initial rate of chondrocyte volume decrease ($\mu m^3.h^{-1}$)	Subsequent rate of chondrocyte volume decrease ($\mu m^3.h^{-1}$)
Control	34.38 ± 0.03	2.87 ± 0.85
Glucosamine	34.50 ± 0.04	1.07 ± 0.05 **
Glucosamine +Rottlerin	13.70 ± 0.30 ***	0.36 ± 0.12 ***
Glucosamine + U73122	6.21 ± 0.03 ***	0.02 ± 0.00 ***
Glucosamine + EGTA	33.48 ± 0.04	0.73 ± 0.14 ***

Figure 4.29: Glucosamine sulphate decreases cell volume by PKC/PLCβ_3 independent mechanisms.

*Cartilage explants were pre-treated for 1 h with control isotonic, glucosamine sulphate (0.1 mg/ml) or glucosamine sulphate with rottlerin (100 μM), U73122 (100 μM) or EGTA (2mM) and subjected to a single mechanical impact. Samples were loaded with Calcein AM (5 μM) and visualised using CLSM prior to and at 2 h, 24 h and 48 h post mechanical impact. Isosurface volume analysis was used to quantify cell volume. Treatment with rottlerin, U73122 and EGTA was not observed to significantly affect initial cell volume compared to glucosamine sulphate controls, but rottlerin and U73122 inhibited the initial rate of impact induced volume decrease. N=12 and n=60 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005 all vs. control. Arrow denotes impact point.*

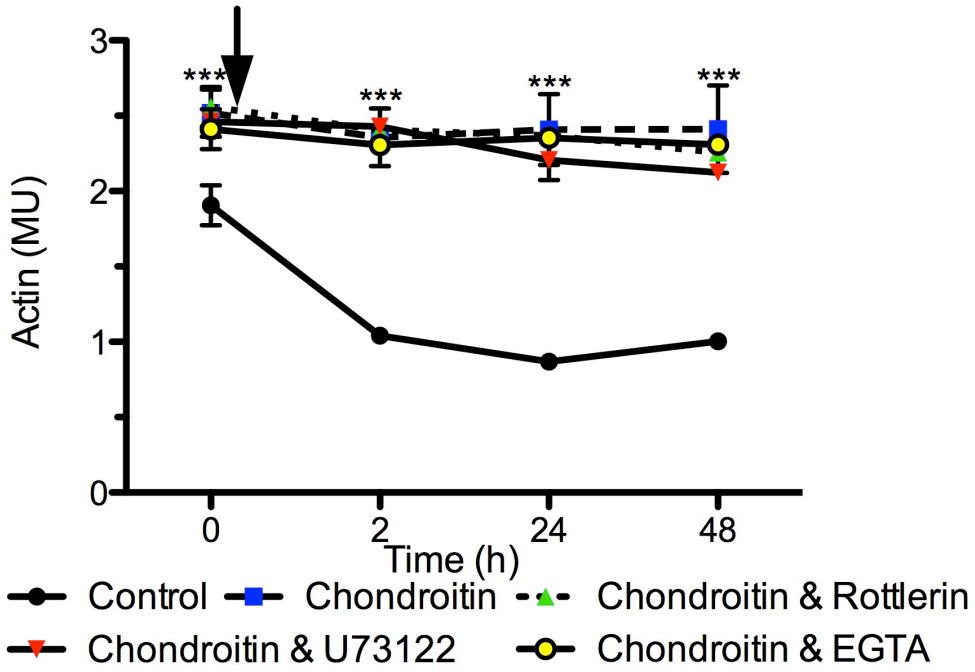
The influence of the PKC/PLCβ_3 signalling pathway on the changes induced by chondroitin and glucosamine sulphate on the F-actin cytoskeleton were investigated, whereby articular cartilage samples were fixed with 4% paraformaldehyde and stained for F-actin with Alexa Fluor 488 Phalloidin (5 μl/ml) prior to and 2, 24 and 48 h post mechanical impact. F-actin was imaged using CLSM and analysed using linear profiling and the PQ Factor, as previously described (*See Methods and Materials*).

Rottlerin, U73122 and EGTA were observed to have no effect on the increase in initial F-actin prior to mechanical impact induced by chondroitin and glucosamine sulphate (*Fig. 4.30/31*). Similarly, rottlerin, U73122 and EGTA exhibited no significant effect ($p>0.05$) on the protection against IIAD observed by chondroitin and glucosamine sulphate (*Fig. 4.30/31*).

Finally, supernatant samples were collected immediately and at 2, 24 and 48 h post mechanical impact and analysed by ELISA for cytokine concentration as previously described (*See Methods and Materials*).

Investigation of inflammatory cytokines revealed no significant changes, compared to chondroitin sulphate treatment, in IL-1β (*Fig. 4.32A*), IL-10 (*Fig. 4.32B*) or MCP-1 (*Fig. 4.32C*) concentration upon additional treatment with rottlerin, U73122 or EGTA. Similarly, no significant effects were seen upon additional treatment with rottlerin, U73122 or EGTA in IL-1β (*Fig. 4.33A*), IL-10 (*Fig. 4.33B*) or MCP-1 (*Fig. 4.33C*) concentration compared to sole treatment with glucosamine sulphate.

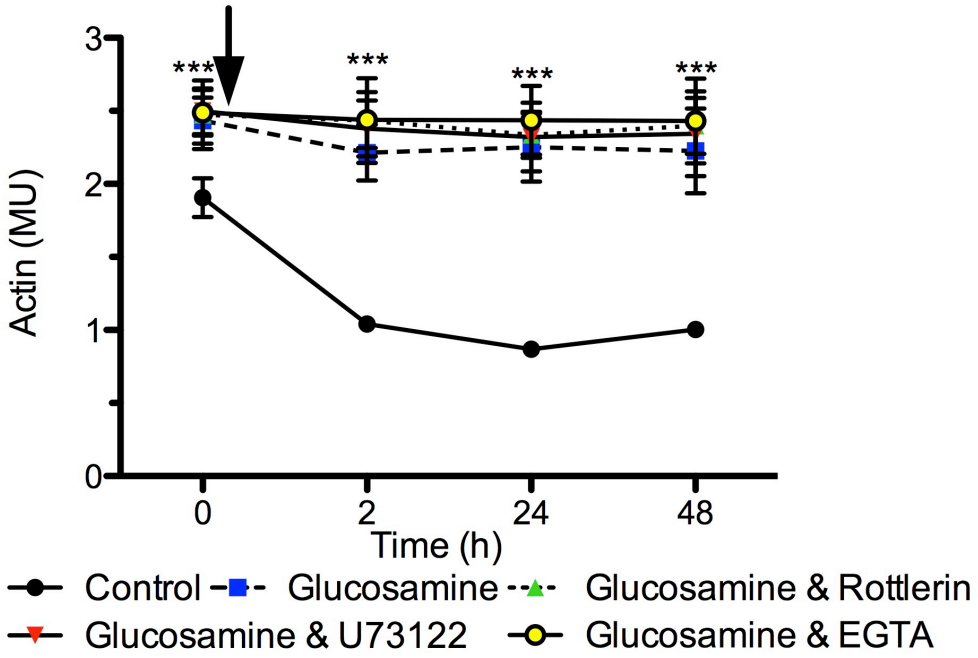
These data demonstrated that the chondroprotective effects of chondroitin and glucosamine sulphate as not mediated by the PLCβ_3/PKC pathway.



Condition	Initial rate of actin polymerisation (MU hour^{-1})	Subsequent rate of actin polymerisation (MU hour^{-1})
Control	-0.43 ± 0.03	0.00 ± 0.00
Chondroitin	-0.08 ± 0.04 ***	0.01 ± 0.01
Chondroitin + Rottlerin	-0.08 ± 0.30 ***	0.01 ± 0.01
Chondroitin + U73122	-0.06 ± 0.05 ***	0.00 ± 0.00
Chondroitin + EGTA	-0.05 ± 0.00 ***	0.01 ± 0.00

Figure 4.30: Chondroitin sulphate's protection against mechanical impact induced decrease in F-actin is PKC/PLCβ_3 independent .

*Cartilage explants were pre-treated for 1 h with control isotonic, chondroitin sulphate (0.3 mg/ml) or chondroitin sulphate with rottlerin (100 μM), U73122 (100 μM) or EGTA (2mM) and subjected to a single mechanical impact. Samples were fixed with 4 % paraformaldehyde and stained with Alexa Fluor 488 phalloidin (5 μl/ml). Cells were visualised using CLSM prior to and at 2 h, 24 h and 48 h post mechanical impact. Linear profiling analysis was used to quantify f-actin, expressed relative to volume changes. Rottlerin, U73122 and EGTA did not significantly affect the increase in f-actin prior to mechanical impact or the protection against impact induced actin decrease observed with chondroitin sulphate. N=12 and n=60 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005 all vs. control. Arrow denotes impact point.*



Condition	Initial rate of actin polymerisation (MU hour^{-1})	Subsequent rate of actin polymerisation (MU hour^{-1})
Control	-0.43 ± 0.03	0.00 ± 0.00
Glucosamine	-0.11 ± 0.04 ***	0.00 ± 0.00
Glucosamine +Rottlerin	-0.02 ± 0.00 ***	0.01 ± 0.00
Glucosamine + U73122	-0.06 ± 0.01 ***	0.00 ± 0.00
Glucosamine + EGTA	-0.03 ± 0.01 ***	0.00 ± 0.00

Figure 4.31: Glucosamine sulphate's protection against mechanical impact induced decrease in F-actin is PKC/PLCβ_3 independent .

*Cartilage explants were pre-treated for 1 h with control isotonic, glucosamine sulphate (0.1 mg/ml) or glucosamine sulphate with rottlerin (100 μM), U73122 (100 μM) or EGTA (2mM) and subjected to a single mechanical impact. Samples were fixed with 4 % paraformaldehyde and stained with Alexa Fluor 488 phalloidin (5 μl/ml). Cells were visualised using CLSM prior to and at 2 h, 24 h and 48 h post mechanical impact. Linear profiling analysis was used to quantify F-actin, expressed relative to volume changes. Rottlerin, U73122 and EGTA did not significantly affect the increase in F-actin prior to mechanical impact or the protection against impact induced actin decrease observed with glucosamine sulphate. N=12 and n=60 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005 all vs. control. Arrow denotes impact point.*

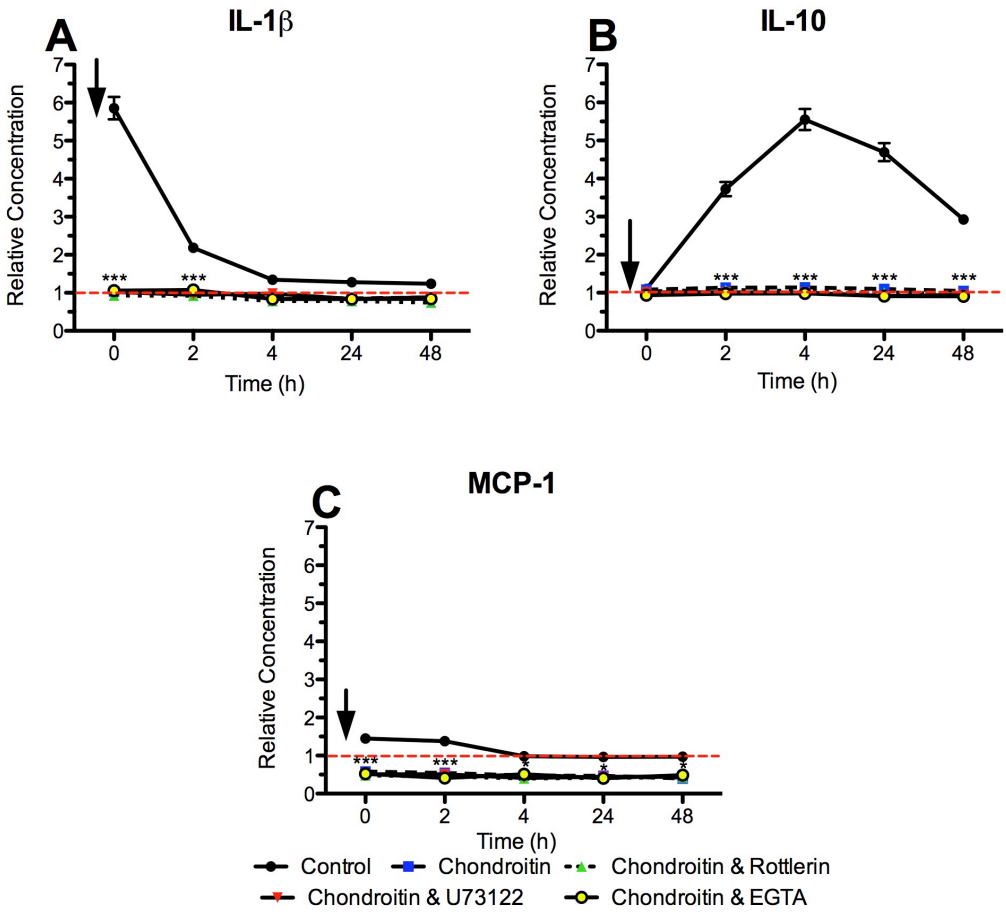


Figure 4.32: Chondroitin sulphate decreases inflammatory cytokines post-mechanical impact independent of PKC/PLCβ$_3$.

*Cartilage explants were pre-treated for 1 h with control isotonic, chondroitin sulphate (0.3 mg/ml),or chondroitin sulphate with rottlerin (100 μM), U73122 (100 μM) or EGTA (2mM) and subjected to a single mechanical impact. Supernatant samples immediately following and at 2 h, 4 h, 24 h and 48 h post mechanical impact were analysed by ELISA and cytokine concentration expressed relative to non-impacted control samples. Treatment with rottlerin, U73122 and EGTA was observed to have no effect on the decrease in IL-1β (A), IL-10 (B) or MCP-1 (C) concentration observed with chondroitin sulphate. N=12 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005. Arrow denotes impact point.*

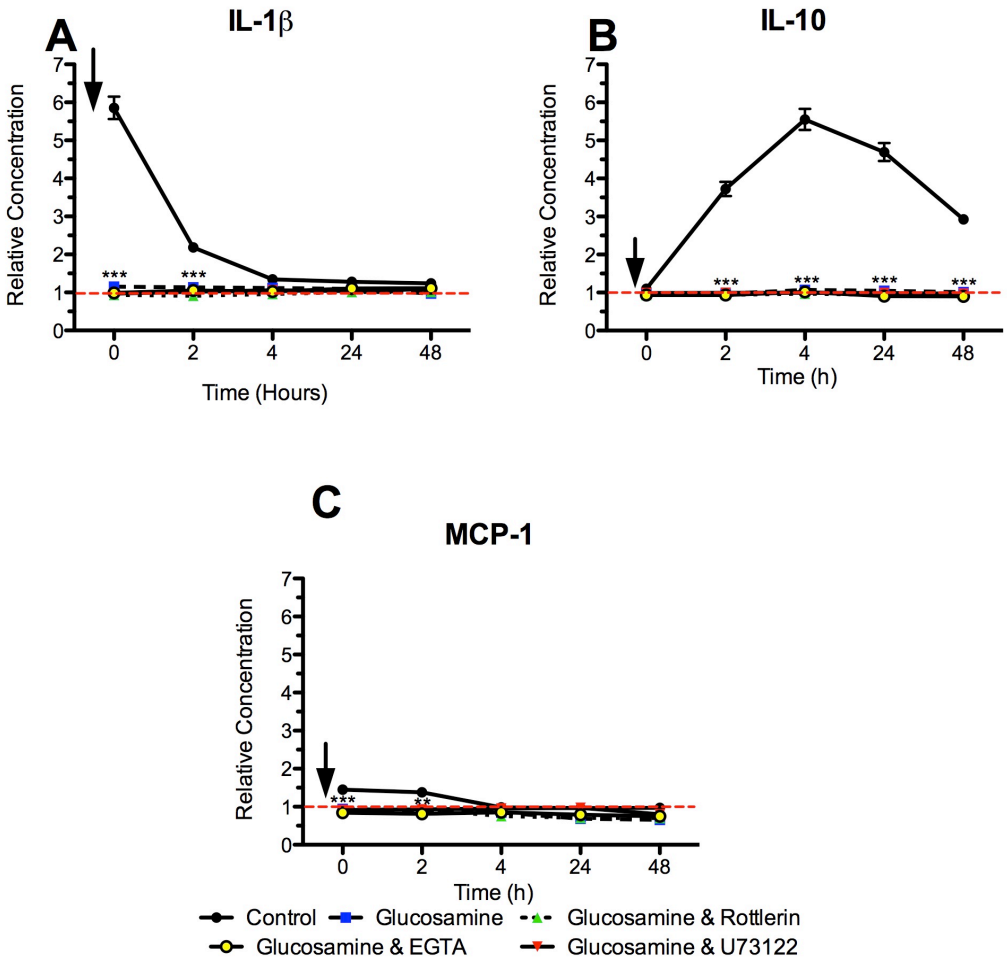


Figure 4.33: Glucosamine sulphate decreases inflammatory cytokines post-mechanical impact independent of PKC/PLCβ_3.

*Cartilage explants were pre-treated for 1 h with control isotonic, glucosamine sulphate (0.1 mg/ml),or glucosamine sulphate with rottlerin (100 μM), U73122 (100 μM) or EGTA (2mM) and subjected to a single mechanical impact. Supernatant samples immediately following and at 2 h, 4 h, 24 h and 48 h post mechanical impact were analysed by ELISA and cytokine concentration expressed relative to non-impacted control samples. Treatment with rottlerin, U73122 and EGTA was observed to have no effect on the decrease in IL-1β (A), IL-10 (B) or MCP-1 (C) concentration observed with glucosamine sulphate. N=12 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005. Arrow denotes impact point.*

4.2.6 Comparison of Chondroprotective Agents

REV 5901, tamoxifen, chondroitin sulphate and glucosamine sulphate all demonstrated chondroprotective effects, whereby they prevented cell death upon mechanical impact. Comparison of the efficiency of the nutriceuticals and pharmaceuticals revealed no significant differences in chondroprotection at 48 h ($p>0.05$). Tamoxifen and chondroitin sulphate were observed to be the most effective at chondrocyte shrinkage prior to mechanical impact, however, tamoxifen was the only compound to prevent IIVD, thus indicating inhibition of IIVD is not essential for chondroprotection. REV 5901 induced the greatest increase in F-action prior to mechanical impact, but all compounds were similarly effective at preventing IIAD. Additionally, REV 5901 was the most efficient at decreasing the inflammatory cytokine response to mechanical impact, reducing peak IL-1β and IL-10 the most, however, chondroitin sulphate decreased MCP-1 more successfully (*Table 4.1*).

	Control	REV 5901	Tamoxifen	Chondroitin Sulphate	Glucosamine Sulphate
Viability at 48 h (%)	45.02±0.99	86.26±3.20	88.30±3.34	89.71±1.98	86.85±4.19
Initial rate of cell death (%.h^{-1})	2.75±0.14	1.50±0.58	0.50±0.03	0.42±0.17	0.67±0.59
Subsequent rate of cell death (%.h^{-1})	0.66±0.05	0.04±0.03	0.12±0.02	0.01±0.01	0.06±0.02
Initial volume (μm^3)	716.14±35.32	552.68±27.26	423.99±15.54	451.09±20.76	523.64±23.41
Initial rate of IIVD ($\mu m^3.h^{-1}$)	34.38±0.03	20.53±0.03	1.19±0.03	37.00±0.07	34.50±0.04
Subsequent rate of IIVD ($\mu m^3.h^{-1}$)	2.87±0.85	0.79±0.06	0.30±0.14	0.71±0.18	1.07±0.05
Initial F-actin (MU)	1.91±0.32	3.20±0.24	2.54±0.07	2.52±0.16	2.43±0.18
IIAD (MU.h^{-1})	0.43±0.03	0.16±0.03	0.07±0.00	0.08±0.00	0.11±0.04
Peak IL-1β (AU)	5.86±0.12	0.75±0.09	2.31±0.16	1.03±0.06	1.05±0.07
Peak MCP-1 (AU)	1.45±0.07	0.67±0.01	1.38±0.16	0.58±0.03	0.98±0.03
Peak IL-10 (AU)	5.55±0.28	0.90±0.17	4.33±0.18	1.14±0.02	1.07±0.06

Table 4.1: The chondroprotective actions of nutriceuticals and pharmaceuticals

A summary of the chondroprotective responses of REV 5901, tamoxifen, chondroitin sulphate and glucosamine sulphate. All agents had a similarly efficient chondroprotective action. REV 5901 was observed to had the greatest impact on decreasing inflammatory cytokine release, and increasing F-actin, however, chondroitin sulphate and tamoxifen had the greatest decrease in initial cell volume. Additionally, tamoxifen was the only agent to inhibit IIVD. IIVD – Impact Induced Volume Decrease, IIAD – Impact Induced Actin Decrease, F-actin – filamentous actin.

4.3 Summary

Investigation of the possible chondroprotective properties of commonly used "healthy joint" promoting nutritional supplements and pharmaceuticals affecting chondrocyte volume regulation or inflammation revealed that REV 5901, Tamoxifen, chondroitin sulphate and glucosamine sulphate all exhibit chondroprotective actions. REV 5901's protective effects were blocked by rottlerin and U73122, indicating them to be dependent upon $[Ca^{2+}]_i$ signalling via the PKC/PLCβ_3 pathway. Conversely, chondroitin and glucosamine sulphate's chondroprotective actions were revealed to be independent of the PKC/PLCβ_3 pathway.

All the investigated agents were observed to decrease cell volume prior to mechanical impact by osmotically independent mechanisms, supporting the observation that cell shrinkage is chondroprotective (*Chapter 3*). IIVD was still observed upon treatment with REV 5901, chondroitin sulphate and glucosamine sulphate but blocked by Tamoxifen, thus suggesting a role for VSOAC within the IIVD mechanism. Inhibition of the PKC/PLCβ_3 pathway by rottlerin and U73122 was observed to inhibit IIVD when used to pre-treat REV 5901, chondroitin sulphate and glucosamine sulphate samples, suggesting a role for $[Ca^{2+}]_i$ signalling via the PKC/PLCβ_3 pathway within IIVD.

F-actin was seen to be increased by REV5901, tamoxifen, chondroitin sulphate and glucosamine sulphate prior to mechanical impact, suggesting increased F-actin concentration prior to mechanical impact is chondroprotective. Conversely, IIAD was not observed in samples treated with REV 5901, tamoxifen, chondroitin sulphate or glucosamine, suggesting a role for IIAD in cell death post mechanical impact.

Finally, REV 5901 was observed to decrease LTB_4 and also decrease inflammatory cytokine production post mechanical impact, suggesting REV 5901's anti-inflammatory actions are mediated by inhibition of 5-LO. Similarly, tamoxifen, chondroitin sulphate and glucosamine sulphate decreased inflammatory cytokines to baseline non-impact values or lower,

independent of the investigated $[Ca^{2+}]_i$ signalling pathway. These data suggest that reduced inflammatory cytokines play a role in the chondroprotective mechanisms of the investigated agents.

To summarise, chondroprotective agents consistently decreased initial chondrocyte volume, increased initial F-actin, protected against IIAD and decreased the inflammatory cytokine response (*Table 4.2*), indicating a role for these within chondroprotection. REV 5901 was the most efficient at increasing initial F-actin, but chondroitin sulphate and tamoxifen induced the greatest decrease in initial volume. Chondroitin sulphate and REV 5901 had the greatest impact on reducing inflammatory cytokine release. Given the unknown safety of REV 5901, chondroitin sulphate is likely to be the most promising chondroprotective agent.

	Chondroprotection
Initial cell volume	↓
IIVD	Possible active cell mechanism for chondroprotection
Initial F-actin	↑
IIAD	Likely to play a role in cell death
Cytokines	↑

Table 4.2: The cellular responses involved in chondroprotection

A summary of the cellular responses that are indicative of chondroprotection. IIVD – Impact Induced Volume Decrease, IIAD – Impact Induced Actin Decrease, F-actin – filamentous actin.

Chapter 5: The Effects of *In Vivo* Joint Forces and Post-Exercise Inflammatory Cytokine Release on Chondrocyte Viability

5.1 Introduction

Whilst exercise participation is acknowledged to be of benefit to general health (Saxon *et al*., 1999), and similarly, mechanical loading, such as exercise activity, is known to be essential for the maintenance of healthy cartilage tissue (Helminen, 1987), the long-term safety of sporting/exercise activity from a musculoskeletal stand-point with respect to osteoarthritis (OA) is less defined. It is noted that continuous stress placed on the joints by long-term and prolonged exercise training may result in microtrauma and degeneration (Moskowitz, 1984), as *in vitro* excessive loading is observed to induce cartilage damage and chondrocyte apoptosis, thus increasing the risk of OA development (Oliveria *et al*., 1999, Tew *et al*., 2000, Pohl *et al*., 2009).

Additionally, exercise has been reported to induce a systemic inflammatory response, with sequential increase in pro-inflammatory and anti-inflammatory cytokines observed (Pedersen *et al*., 1998). Increased release of pro-inflammatory cytokines are characteristic of OA, and upregulation in these leads to degradation of the ECM and chondrocyte apoptosis (Rowan and Young, 2007, Westacott and Sharif, 1996). The magnitude of the inflammatory response to exercise is observed to differ depending on the intensity of the exercise undertaken (Bruunsgaard *et al*., 1997, Nieman *et al*., 1998, Moldoveanu *et al*., 2001), suggesting increased joint forces may play a role in elevating inflammation, as higher intensity exercise tends to also be higher impact.

Foot strike patterns, when running are known to vary, with no distinctive style being confirmed as ‘best’. In terms of sporting performance, there are advocates for the ‘pose’ style of running, a mid-forefoot strike pattern, claiming better speed, and conversely, advocates for the heelstrike style of running, claiming greater stride length (Hasegawa *et al*., 2007, Lieberman *et al*., 2010). Thus, sprinters tend to favour forefoot striking, and 75-80 % of elite endurance runners are reported to favour heelstriking (Hasegawa *et al*., 2007, Lieberman *et al*., 2010). Lower limb injury is prevalent amongst runners, with 78.9 % of runners experiencing lower limb injury within 7 days

post marathon, with the knee being the most injured site (Satterthwaite *et al*., 1996). Given that the lower limb experiences forces equivalent to 2-3 times bodyweight per foot strike (Bobbert *et al*., 1991, Chan and Rudins, 1994, Dufek and Bates, 1990, Farley and Gonzalez, 1996, James *et al*., 1978), and that the average endurance runner strikes the ground 600 times per km (Milner *et al*., 2006, Pohl *et al*., 2009), this is unsurprising. As yet, no definitive reports of which foot strike pattern is most effective at injury risk reduction, although there is evidence to suggest that heelstriking increases the force the lower limb joints are subjected to (Sol, 2001).

Given the links between strike pattern and joint forces, and joint forces and inflammatory cytokine release, and the role that uncontrolled inflammation can play in the development OA, it is of interest to investigate the possible links between strike pattern and alterations in pro and anti-inflammatory cytokine levels. Furthermore, it is conceivable that there is a link between the diurnal nature of cartilage (*Chapter 3*) and the known involvement of inflammatory cytokines in cartilage degradation, suggesting a possible diurnal inflammatory response to exercise/trauma.

Systemic inflammation is a multicellular and multi-tissue process. As such inflammation within a single tissue, such as articular cartilage, has multiple contributors. Within the synovial fluid are a variety of cell types, including polymorphonuclear granulocytes (PMNs) and mononuclear phagocytes, such as macrophages (Freemont, 1991). Macrophages in culture have been observed to produce a number of both pro and anti-inflammatory cytokines, including IL-1β and IL-10, in addition to matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs; (Bondeson *et al*., 1999, Amos *et al*., 2006), and have been identified in abundance within OA tissue (Benito *et al*., 2005, Farahat *et al*., 1993, Haywood *et al*., 2003). This suggests a role for mononuclear phagocytes within cartilage inflammation, and thus ECM degeneration and the potential onset of OA.

Given the widely reported role of inflammatory cytokines within the OA pathogenesis, as mediators of ECM degradation and chondrocyte

apoptosis (Haywood *et al*., 2003, Goldring *et al*., 2008), the potential exists for unravelling the cascade of events that ensues following impact trauma and the role that anti-inflammatory agents could play in chondroprotection. Agents such as Curcumin, the naturally occurring active polyphenolic component of the powdered turmeric (*Curcuma longa*) root, have been reported to possess potent anti-inflammatory and anti-oxidant properties (Sharma *et al*., 2005). These effects include down-regulation of MCP-1 expression, most likely through differential regulation of PKC, which in turn is reported to be an upstream signaling molecule of MMP-9, a ECM degrading enzyme (Wu *et al*., 2006, Herman *et al*., 2009). Thus, it is suggested curcumin has both anti-inflammatory and anti-catabolic actions, both of which have the potential to be chondroprotective. The glucocorticoid dexamethasone, is a potent anti-inflammatory drug used to treat a large number of inflammatory and autoimmune disorders including rheumatoid arthritis. Thus, dexamethasone and curcumin are investigated here as possible chondroprotective agents.

5.1.1 Chapter Aims

This study aims to investigate the role of joint forces and alterations in pro and anti-inflammatory cytokines at a systemic level in participants and at the joint and tissue level in bovine cartilage in conjunction with chondrocyte death post mechanical trauma. Specifically by:

1. Investigation of the relationship between knee joint force and inflammatory cytokine production.
2. Study of the effects of strike pattern on knee joint force and inflammatory cytokine production.
3. Elucidation of the possible diurnal nature of the inflammatory response post exercise.
4. Determination of the role of synovial mononuclear phagocytes within the inflammatory response to mechanical impact in articular cartilage.
5. Investigation of the role of inflammatory cytokines in chondrocyte death and the possibilities of manipulating this for chondroprotective purposes.

5.2 Results

5.2.1 Maximal Knee Force

Participants were recruited within a defined age group (20-39 yrs), weight (65-75 kg), percentage body fat (10-13 %) and thigh circumference (48-53 cm). GRF data was collected via forceplate, and leg anthropometry used to calculate joint forces through inverse dynamics (Hutchinson, 1994). Controlling weight, percentage body fat and thigh circumference resulted in an average population barefoot running knee force ± less than 7% (*Table 5.1*).

GRF data was used to calculate ankle, knee and hip joint forces when running both barefoot and in running shoes. It was observed that joint forces were not significantly different between joints, but all were significantly increased ($p<0.05$) when running barefoot, whereby ankle, knee and hip force all increased by 13 % when running barefoot (*Fig. 5.1*).

When comparing maximal dominant leg (MDL) knee force whilst running, no correlation was observed between barefoot and when wearing running shoes, despite population data revealing a 13 % increase in joint forces when running barefoot compared to in shoes (Fig. 5.1), thus demonstrating footwear had a non-standard effect on knee force (*Table 5.2*). Additionally, maximal knee force when running barefoot was compared between dominant and non-dominant legs, and again no correlation was observed, indicating a non-standardised effect of leg dominance on knee force (*Table 5.2*). Based on these data, MDL knee force when running barefoot was used henceforth.

The possible association between joint force and systemic circulating inflammatory cytokine concentration was investigated, whereby circulating cytokines were analysed by ELISA from venous blood samples obtained immediately post 30 min treadmill exercise at 60 % VO_2max and standardised to pre-exercise levels, as previously described (*See Materials and Methods*). MDL knee force when running barefoot was determined from GRF data and compared to changes in circulating cytokines.

Circulating pro-inflammatory cytokines IL-1β and MCP-1 were observed to associate positively with MDL knee force when running barefoot, with a Pearson correlation coefficient (r^2) of 0.85 and 0.70 revealed respectively. Additionally, the circulating anti-inflammatory cytokine IL-10 was observed to significantly ($p<0.05$) correlate with MDL knee force, whereby an r^2 of 0.89 was noted (*Fig. 5.2*). These data suggested a role for increasing joint forces in increased systemic circulating cytokine concentration post exercise.

	Average ± SEM
Age (Years)	34.63 ± 2.16
Weight (Kg)	69.89 ± 3.34
Height (cm)	173.14 ± 2.89
Body Fat (%)	11.60 ± 1.19
BMI	22.12 ± 2.63
Thigh Circumference	52.1 ± 3.45
Maximum Knee Force (Barefoot)	3935.98 ± 257.01
UK Shoe Size	9 ± 0.61
Sole Depth (cm)	3.28 ± 0.30

Table 5.1: Subject characteristics summary

A homogenous subject population was recruited, controlled for age (20-39 yrs), weight (65-75 kg), body fat percentage (10-13 %) and thigh circumference (48-53 cm) to standardise resultant dominant barefoot kneeforce. Data from N=14 individuals.

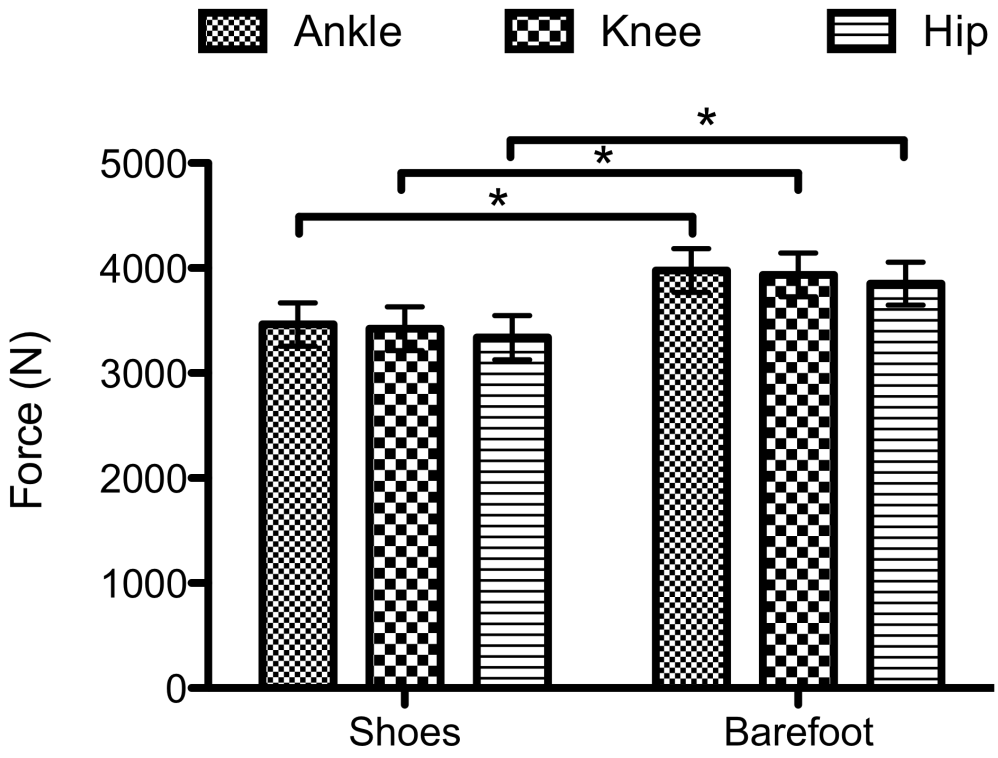


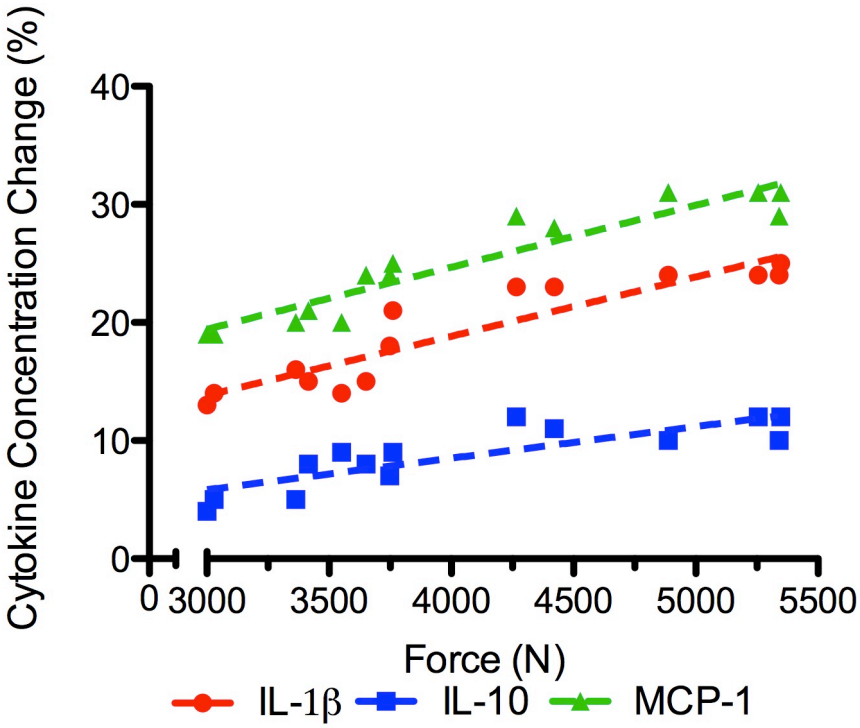
Figure 5.1: Dominant leg joint forces are decreased by footwear.

*GRF forceplate data was used to calculate dominant leg joint forces whilst running, both barefoot and in running shoes. Joint forces were observed to be significantly decreased when wearing running shoes. Data from N=14 individuals.*p<0.05, **p<0.01, ***p<0.005 vs shoes.*

	Correlate (Y/N)	P Value	Pearson Coefficient
Barefoot Vs Shoes	N	0.34	0.08
Dominant Vs Non-dominant	N	0.30	0.12

Table 5.2: Effect of footwear and leg dominance on knee force.

GRF forceplate data was used to calculate dominant and non-dominant leg knee forces whilst running, both barefoot and in running shoes. Data are from N=14 individuals.



	Correlate (Y/N)	P Value	Pearson Coefficient
Maximal Force Vs Relative IL-1β	Y	0.001	0.85
Maximal Force Vs Relative IL-10	Y	0.001	0.70
Maximal Force Vs Relative MCP-1	Y	0.001	0.89

Figure 5.2: Effect of elevated Knee force on systemic pro and anti-inflammatory cytokines.

GRF forceplate data was used to calculate dominant leg knee forces whilst running barefoot. Circulating blood cytokines were analysed by ELISA from venous blood samples obtained immediately post 30 min treadmill exercise at 60 % VO_2max. Cytokine concentrations are expressed relative to resting values obtained prior to exercise. Data is from N=14 participants measured in triplicate.

5.2.2 The Influence of Strike Pattern on Knee Force

The varying strike pattern of participants was analysed to elucidate any links between strike pattern and maximal knee force, whereby strike pattern was determined by mapping maximal GRF during each footstrike (*Fig. 5.3*) and calculating a ratio of maximal heelstrike:ball GRF and pronation:supernation as previously described (*See Materials and Methods*).

MDL knee force when running barefoot was observed to have a significant ($p<0.05$) correlation with heelstrike:ball, identified by an r^2 of 0.91 (*Fig. 5.4*). In addition, pronate:supernate was seen to induce an r^2 of 0.85 with MDL knee force when running barefoot (*Fig. 5.5A*), suggesting that pronated and heelstrike gaits induced greater knee forces when running.

Given the previous observation that increased knee forces were associated with increased systemic circulating cytokines, strike patterns were correlated with maximal circulating cytokines. It was observed that pronate:supernate associated positively with increasing circulating cytokines, whereby r^2 values of 0.82, 0.73 and 0.86 were computed when pronate:supernate was compared with maximal IL-1β, IL-10 and MCP-1 respectively (*Fig. 5.5B*). Conversely, despite increasing heelstrike being associated with increased knee force, heelstrike:ball was not observed to correlate with circulating cytokine levels of IL-1β, IL-10 or MCP-1 (*Fig. 5.4*).

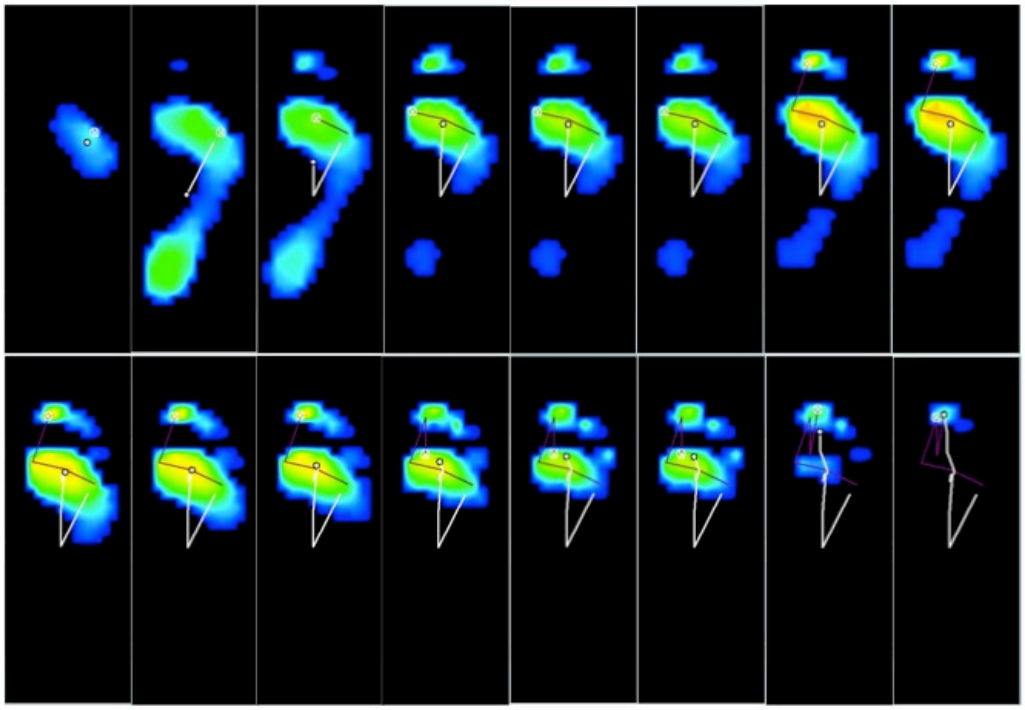
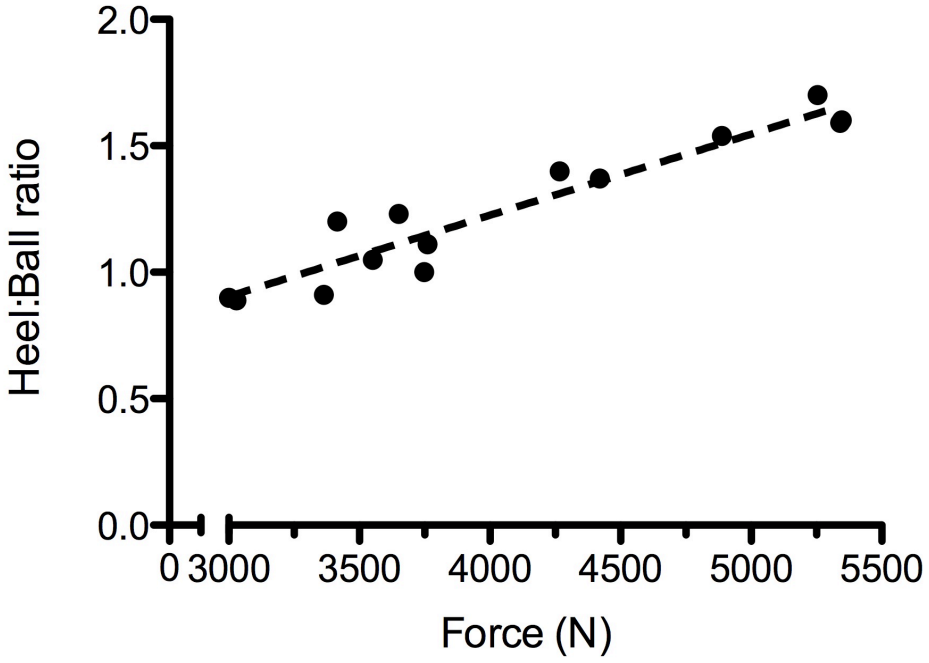


Figure 5.3: Representative running forceplate GRF data images.

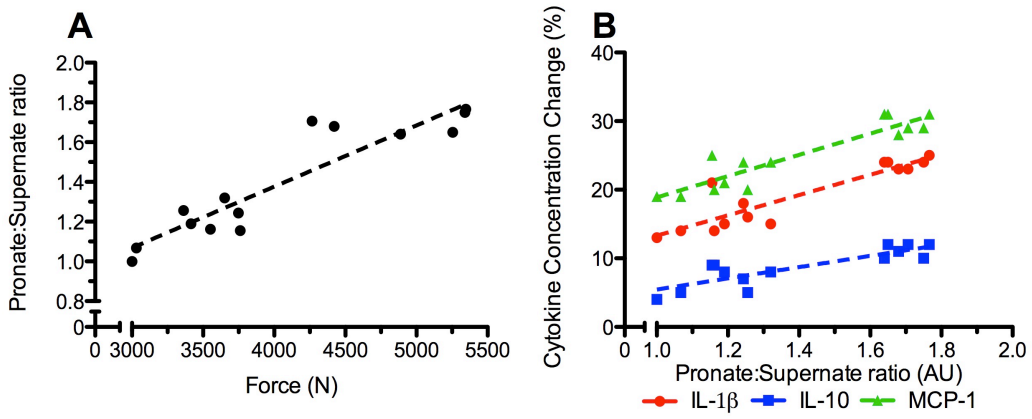
Participants ran across the forceplate at an approximate long distance running pace (13.4 km.h^{-1}) while maximal GRF (⊗, mapped by the white line) and centre of mass (°, mapped by the purple line) were measured.



	Correlate (Y/N)	P Value	Pearson Coefficient
Maximal Force Vs H:B	Y	0.001	0.91
H:B Vs Relative IL-1β	N	0.22	0.49
H:B Vs Relative IL-10	N	0.42	0.33
H:B Vs Relative MCP-1	N	0.59	0.23

Figure 5.4: Effect of Heel-striking gait on maximal knee force.

GRF forceplate data was used to calculate dominant leg knee forces whilst running barefoot and analyse heel-strike gait as a ratio of maximal GRF at the heel and maximal GRF at the ball. Circulating blood cytokines were analysed by ELISA from venous blood samples obtained immediately post 30 min treadmill exercise at 60 % VO_2max. Cytokine concentrations are expressed relative to resting values obtained prior to exercise. Data are from. N=14 participants measured in triplicate.



	Correlate (Y/N)	P Value	Pearson Coefficient
Maximal Force Vs P:S	Y	0.001	0.85
P:S Vs Relative IL-1β	Y	0.001	0.82
P:S Vs Relative IL-10	Y	0.002	0.73
P:S Vs Relative MCP-1	Y	0.001	0.86

Figure 5.5: Effect of Pronated gait on maximal knee force and circulating inflammatory cytokines.

GRF forceplate data was used to calculate dominant leg knee forces whilst running barefoot and analyse pronated gait as a ratio of maximal GRF at the medial and lateral aspects of the ball of the foot. Circulating blood cytokines were analysed by ELISA from venous blood samples obtained immediately post 30 min treadmill exercise at 60 % VO_2max. Cytokine concentrations are expressed relative to resting values obtained prior to exercise. Pronate:Supernate ratio was seen to significantly correlate with both force (A) and circulating inflammatory cytokine changes (B). Data are from N=14 participants measured in triplicate.

5.2.3 Diurnal Exercise and Inflammation

Following investigation of the effects of maximal knee force and strike pattern on knee force, alterations in early morning and evening circulating cytokines were analysed by ELISA from venous blood samples obtained prior to, immediately post and at 2 and 4 h post 30min treadmill exercise, undertaken at either 8am (AM) or 6pm (PM) and at an intensity of 60 % VO_2max.

It was observed that both the pro-inflammatory cytokines IL-1β and MCP-1 relative concentration increased post exercise for both AM (*Fig. 5.6*) and PM exercise groups (*Fig. 5.6*), peaking immediately post exercise before decreasing. Peak IL-1β relative concentration post exercise was observed to be increased by 20.67±2.58 % after AM exercise, but only by 9.60±2.35 % after PM exercise (*Fig. 5.6A*). Similarly, MCP-1 relative concentration was seen to be increased after AM exercise when compared to PM exercise, whereby AM exercise increased by 25.69±0.53 %, and PM exercise by 9.01±2.94 % (*Fig. 5.6C*). Following observations that exercise increased pro-inflammatory cytokine concentration, measurement of the anti-inflammatory cytokine IL-10 was determined. IL-10 relative concentration was seen to increase post exercise in both AM and PM exercise groups, peaking at 2 h post exercise, with no significant difference observed between the AM exercise peak increase of 8.97±2.40 % and the PM exercise peak increase of 10.08±2.30 % (*Fig. 5.6B*).

These data suggested a diurnal role for circulating cytokines post exercise, whereby PM exercise was associated with decreased systemic pro-inflammatory cytokine concentration when compared to AM exercise.

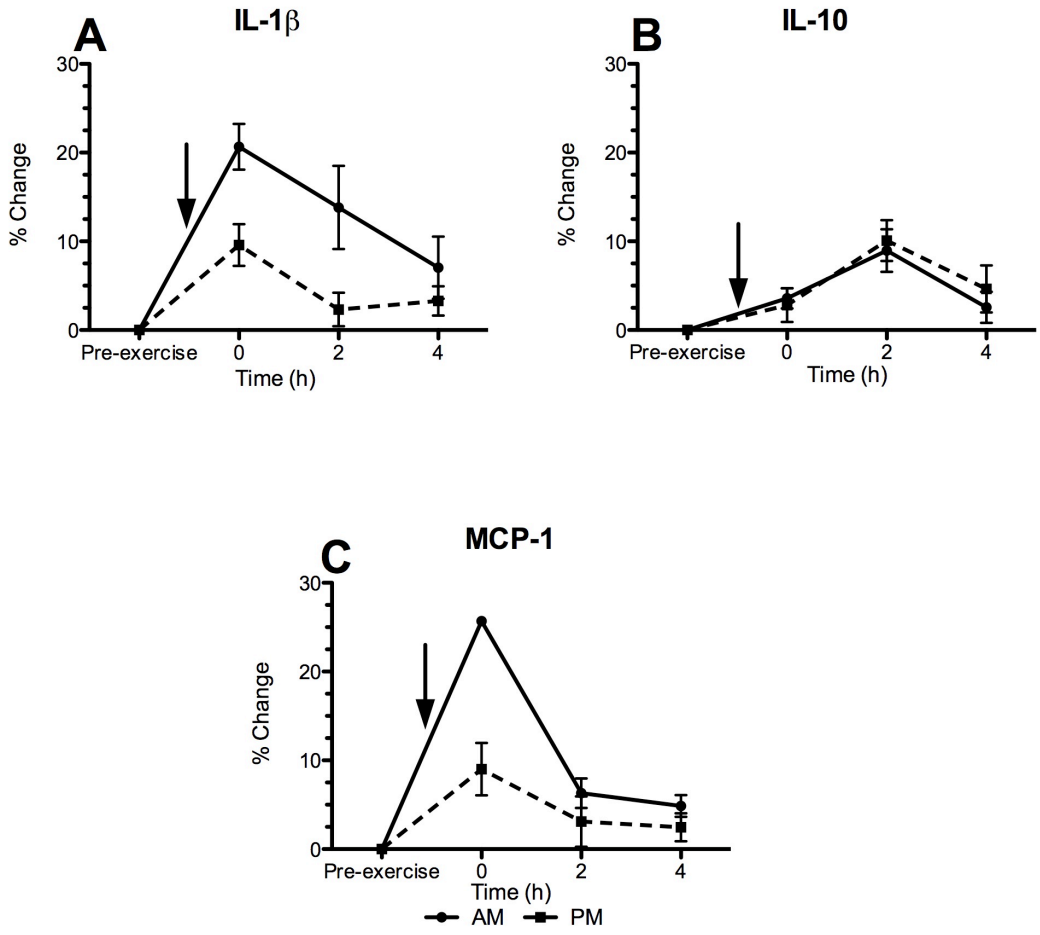


Figure 5.6: Determination of systemic IL-1β, IL-10 and MCP-1 levels post exercise.

Circulating blood cytokines were analysed by ELISA from venous blood samples obtained immediately, 2 and 4 h post 30 min treadmill exercise at 60 % VO_2max. Exercise was undertaken at 8am (AM) or 6pm (PM). Cytokine concentrations are expressed relative to resting values obtained prior to exercise. Peak circulating concentration was observed for both IL-1β (A) and MCP-1 (C) immediately post exercise for both AM and PM exercise, whereas peak circulating concentration was observed for IL-10 (B) at 2 h post exercise for both AM and PM exercise. Peak pro-inflammatory cytokine concentration, both IL-1β (A) and MCP-1 (C), was observed to be increased post AM exercise when compared to PM exercise. Conversely, no significant difference was observed in peak anti-inflammatory cytokine concentration (IL-10, B) post exercise between AM and PM exercise. Data are mean ± s.e.m from N=14 participants measured in triplicate. Arrow denotes exercise point.

5.2.4 Articular Cartilage Cytokine Release and Mononuclear Phagocytes

Following the identification that impact led to significant increases in the release of pro and anti-inflammatory cytokines, supernatants from these impacted tissue were used to activate isolated synovial mononuclear phagocytes.

Articular cartilage explants subjected to a single mechanical trauma were observed to release increased concentrations of IL-1β, IL-6, IL-8, MCP-1 and IL-10 (*Fig. 5.7A*) into the supernatant, whereby IL-1β increased from 33.53±4.21 to 73.23±2.28 pg/ml/g, IL-6 increased from 2.50±0.22 to 110.84±2.99 pg/ml/g, IL-8 increased from 35.46±1.88 to 100.79±4.61 pg/ml/g, MCP-1 increased from 50.02±6.44 to 68.98±4.23 pg/ml/g and IL-10 increased from 80.98±24.31 to 301.75±29.34 pg/ml/g.

Bovine mononuclear phagocytes were isolated from synovial fluid, whereby other synovial cells were removed by trypsin, and the remaining trypsin-resistant mononuclear phagocytes stimulated with supernatants from explants immediately post mechanical trauma, as previously noted to be the peak pro-inflammatory cytokine time-point. Mononuclear phagocyte supernatants were analysed by ELISA prior to and at 1 h post stimulation. Mononuclear phagocytes were observed to produce minimal concentrations of IL-1β, IL-6, IL-8, MCP-1 and IL-10 prior to stimulation, ranging from 23.04±4.22 pg/ml of IL-8 to 113.05±12.48 pg/ml of IL-10 (*Fig. 5.7B*). Stimulation with supernatants from impact explants was observed to induce a significant ($p<0.01$) increase in pro-inflammatory cytokines IL-1β and MCP-1 from 65.51±9.13 pg/ml and 90.23±11.38 pg/ml to 219.90±23.40 pg/ml and 366.74±27.88 pg/ml respectively (*Fig. 5.7B*). Furthermore a significant ($p<0.01$) decrease in anti-inflammatory cytokine IL-10 concentration of approximately 80 % was induced by mononuclear phagocyte stimulation to 23.07±3.62 pg/ml.

Bovine mononuclear phagocytes were co-cultured with explants subjected to mechanical trauma and supernatants analysed by ELISA at 1 h post co-culture. Resultant cytokine concentration was adjusted to reflect the concentration solely produced by the articular cartilage by subtraction of the previously observed cytokine concentrations produced by baseline and stimulated mononuclear phagocytes. It was noted that articular cartilage co-cultured with mononuclear phagocytes produced significantly increased concentrations of IL-1β compared to both baseline cartilage and cartilage subjected to mechanical trauma, whereby IL-1β concentration was increased by 118 % by mechanical impact, and by a further 142 % upon co-culture with mononuclear phagocytes (*Fig. 5.7C*). The expression of IL-6, IL-8, MCP-1 and IL-10 was not observed to be significantly ($p>0.05$) altered upon co-culture.

These data suggest a role within the impact induced inflammatory cytokine response, of a possible paracrine positive-feedback loop between articular cartilage and mononuclear phagocytes, driven by IL-1β.

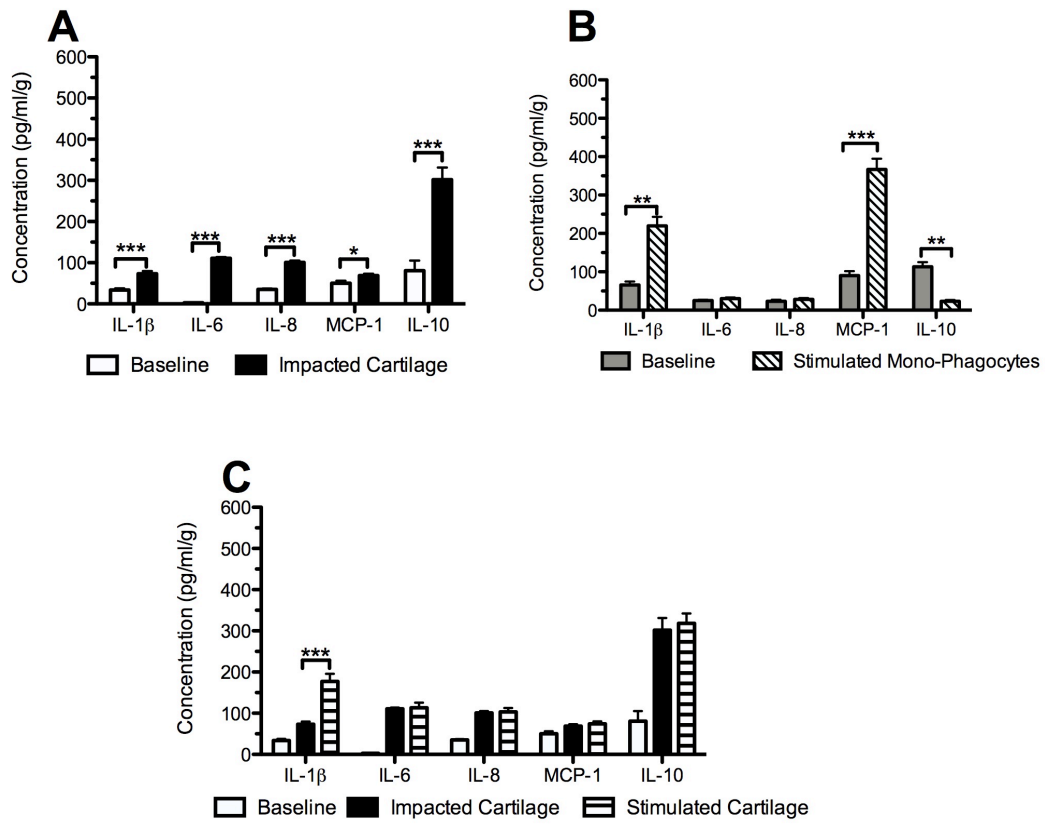


Figure 5.7: Inflammatory cytokine concentrations released by articular cartilage and mononuclear phagocytes upon stimulation by single mechanical impact

*(A)Supernatants from control explants and explants submitted to a single mechanical insult were analysed at immediately post-impact by ELISA to determine cytokine concentration. It was observed that upon impact there was a significant increase in concentration of pro-inflammatory cytokines (IL-1β, IL-6, IL-8 and MCP-1) in addition to an increase in anti-inflammatory cytokine concentration (IL-10). (B)Supernatants from control mononuclear phagocytes and mononuclear phagocytes stimulated with supernatant from impacted explants were analysed 1 h post-stimulation by ELISA. Stimulation with supernatant from impacted cartilage containing increased concentrations of inflammatory cytokines was seen to significantly increase the release of IL-1β and MCP-1 from mononuclear phagocytes. (C)Supernatants from control, explants submitted to a single impact and explants submitted to a single impact and incubated with mononuclear phagocytes were analysed by ELISA at 1 h. A significant increase in the concentration of IL-1β was observed. N=12 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005.*

5.2.5 The Role of Inflammatory Cytokines in Chondrocyte Death Post Mechanical Impact

Following observation of increased inflammatory cytokine release by articular chondrocytes in response to mechanical impact, the role of cytokines within impact induced cell death was investigated, whereby supernatants taken immediately post mechanical impact from explants pre-treated with isotonic (280 mOsm), hyperosmotic (380 mOsm) or hypo-osmotic (140 mOsm) DMEM were applied to non-impacted explants and samples were analysed by CLSM for viability at 1 h post incubation.

Supernatants from samples pre-treated with isotonic and hypo-osmotic DMEM were observed to induce a decrease in cell viability from non-impact control levels of 94.28±2.76 % to 75.13±3.26 % and 73.02±2.12 % respectively (*Fig. 5.8*). Conversely, supernatants from explants pre-treated with hyperosmotic DMEM did not induce significant cell death. As hyperosmotic challenge was previously observed to decrease the cytokine release response to impact, these data suggested a role for the inflammatory cytokines released into the supernatant in response to impact in cell death.

As previous data suggested a role for IL-1β in mediating the inflammatory cytokine response (*Section 5.2.4*), the role of IL-1β in cytokine induced cell death was investigated by direct media supplementation with 200 pg/ml IL-1β per gram of cartilage, the equivalent concentration of IL-1β released by articular cartilage in response to impact. Samples were analysed by CLSM for viability and volume post 1 h incubation. Treatment with IL-1β (*Fig. 5.9A)* was observed to induce significant cell death, whereby cell viability was decreased from 94.28±2.76 % prior to IL-1β stimulation to 75.98±3.80 % (*Fig. 5.9C*). Furthermore, IL-1β treatment (*Fig. 5.10A)* was observed to significantly decrease cell volume by 30 % to 409.30±13.08 μm^3 (*Fig. 5.10C*). These data suggest IL-1β is a contributor to cytokine mediated cell death, and potentially plays a significant role in cell death post impact.

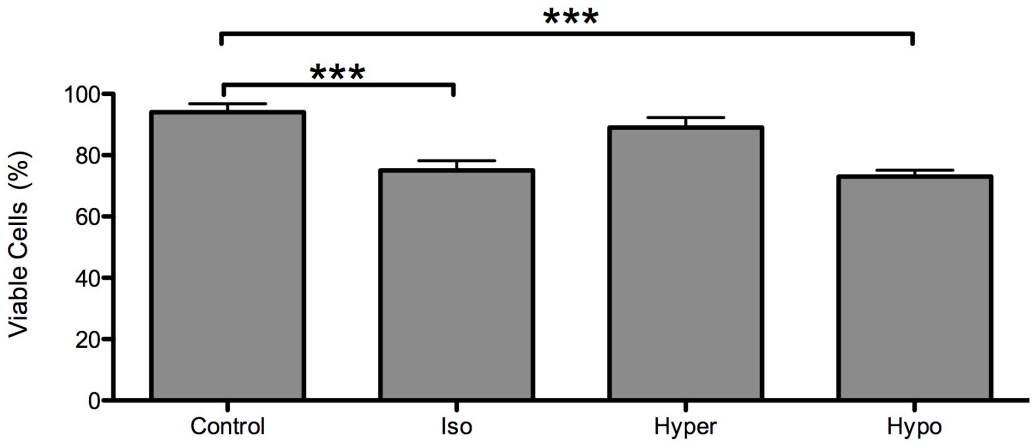


Figure 5.8: Stimulation with supernatants from impacted explants decreases cell viability.

*Cartilage explants were pre-treated for 1 h with control isotonic DMEM or supernatants from explants immediately post impact after 1 h pre-treatment with control isotonic, hyperosmotic (380 mOsm) or hypo-osmotic (140 mOsm) DMEM. Samples were stained with Calcein AM (5 μM) and PI (1 μM) and visualised using CLSM at post stimulation. Imaris spot analysis was used to quantify cell viability. Stimulation with supernatants from impacted explants pre-treated with control and hypo-osmotic DMEM was observed to significantly decrease cell viability compared to control non-impacted samples. Conversely, stimulation with supernatants from impacted explants pre-treated with hyperosmotic DMEM did not significantly effect viability. N=12 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005*

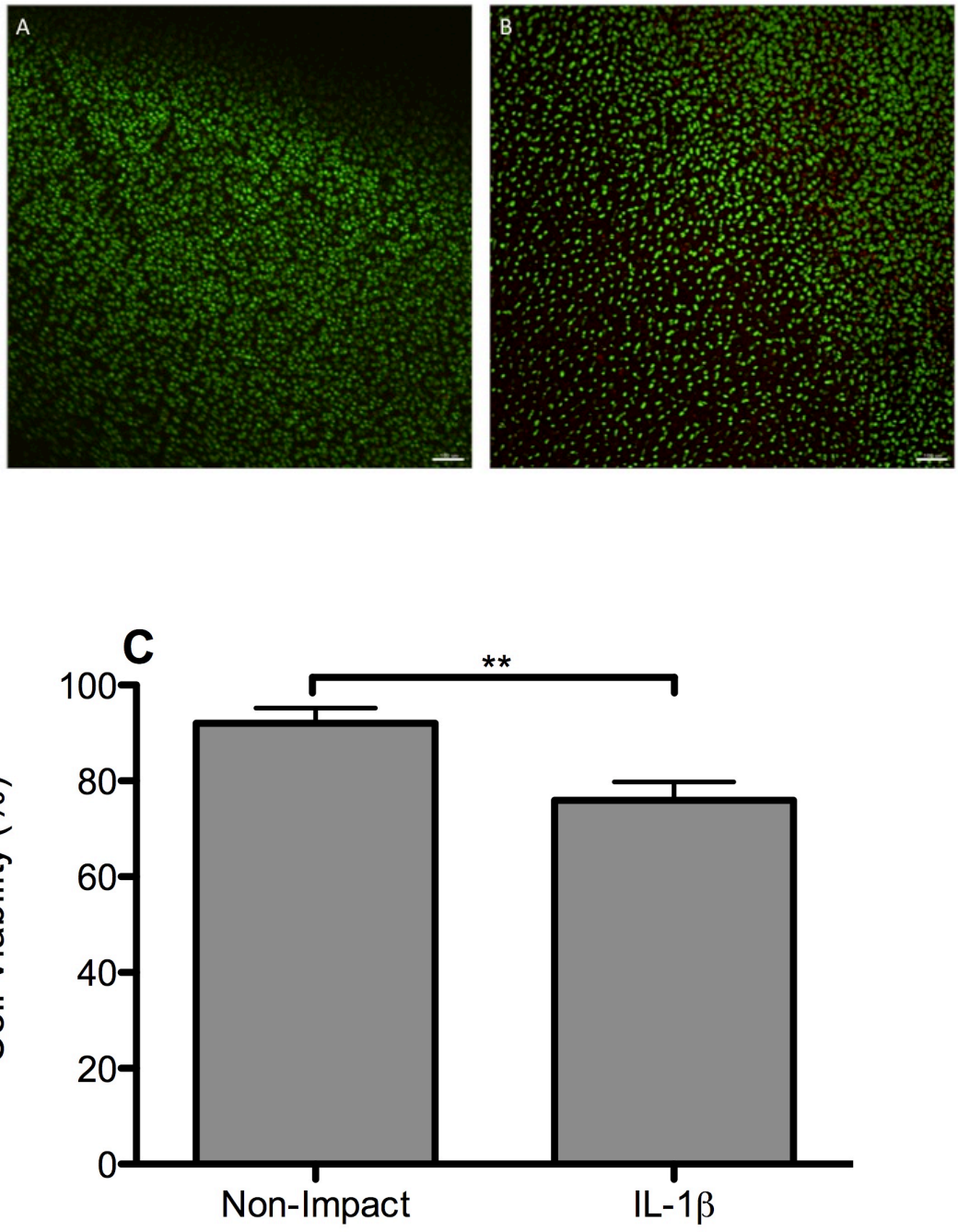


Figure 5.9: IL-1β stimulation induces cell death

*Cartilage explants were pre-treated for 1 h with control isotonic (A) or IL-1β (200 pg/ml/g; B) supplemented DMEM. Samples were stained with Calcein AM (5 μM) and PI (1 μM) and visualised using CLSM post stimulation. Imaris spot analysis was used to quantify cell viability. IL-1β stimulation was observed to significantly decrease cell viability compared to non-impact controls. N=12 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005*

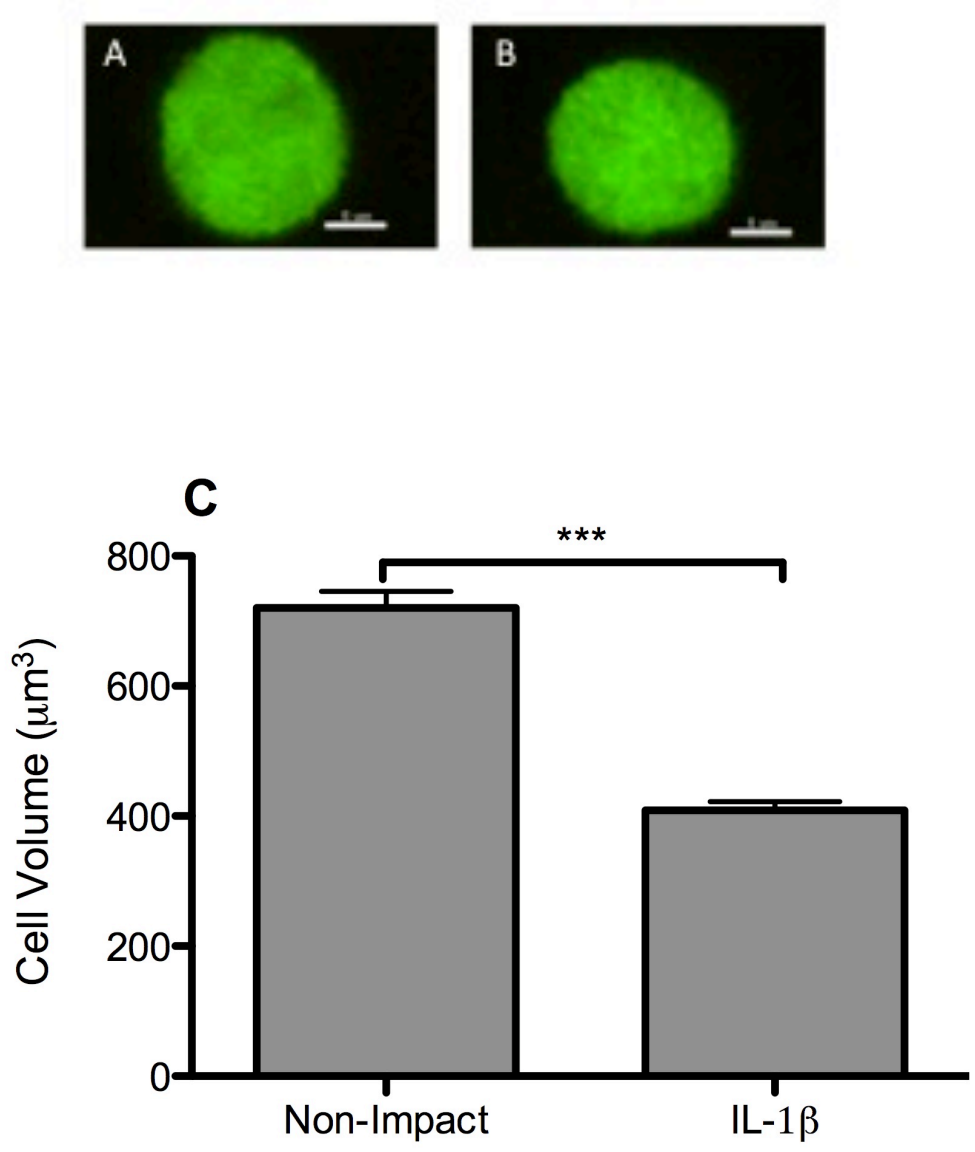


Figure 5.10: IL-1β stimulation induces cell death and decreases cell volume

*Cartilage explants were pre-treated for 1 h with control isotonic (A) or IL-1β (200 pg/ml/g; B) supplemented DMEM. Samples were stained with Calcein AM (5 μM) and visualised using CLSM post stimulation. Isosurface volume analysis was used to quantify cell volume. IL-1β stimulation was observed to significantly decrease cell volume (C). n=60 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005*

5.2.6 Effect of Curcumin and Dexamethasone on Pro and Anti-Inflammatory Cytokine Release and Chondroprotection.

Given that the direct addition of IL-1β as well as impact caused a significant increase in cell death, the protective effects of the anti-inflammatory agents curcumin (50 μM) and dexamethasone (1 μM) were subsequently evaluated. Specifically, the release of IL-1β, MCP-1, IL-10 were determined by ELISA and chondrocyte viability by CLSM as previously described.

In response to mechanical impact, an increase in the release of IL-1β, MCP-1 and IL-10 were observed, whereby IL-1β and MCP-1 increased immediately post impact to 5.86±0.29 AU and 1.45±0.07 AU respectively, before declining down to baseline levels (1 AU). IL-10 was observed to additionally increase post impact, peaking at 24 h at 5.55±0.28 AU (*Fig. 5.11*).

Curcumin and dexamethasone were confirmed to have immunomodulatory actions, whereby a prevention of IL-1β increase in response to impact was observed. IL-1β concentration immediately post impact was decreased to 1.02±0.01 AU and 0.92±0.04 AU by curcumin and dexamethasone respectively. Subsequently, curcumin and dexamethasone induced a decrease in IL-1β release to 0.66±0.02 AU and 0.65±0.03 AU by 48 h respectively (*Fig. 5.11A*). Furthermore, peak IL-10 observed at 24 h was decreased from 5.55±0.28 AU to 1.27±0.06 AU and 1.24±0.05 AU by curcumin and dexamethasone (*Fig. 5.11B*) and peak MCP-1 observed at 0 h was decreased to 1.11±0.10 AU by dexamethasone and to below non-impact levels (1 AU) by curcumin to 0.95±0.03 AU where it subsequently decreased to a low of 0.40±0.09 AU by 48 h (*Fig. 5.11C*).

Having confirmed the antagonistic effects of curcumin and dexamethasone on the inflammatory cytokine response to impact, the possible chondroprotective effects of curcumin and dexamethasone were evaluated. Whilst neither agent was observed to significantly ($p>0.05$) effect the initial rate of chondrocyte death following impact, both curcumin and

dexamethasone significantly ($p<0.005$) reduced the subsequent rate of death, whereby the rate of death was decreased from 0.66±0.03 $\%.h^{-1}$ to 0.07±0.02 $\%.h^{-1}$ and 0.13±0.09 $\%.h^{-1}$ respectively. This duly resulted in an overall chondroprotective effect with cell viability at 48 h post impact increased from 45.02±2.52 % to 82.58±3.12 % by curcumin and 79.68±3.06 % by dexamethasone (*Fig. 5.12*).

These data suggest that suppression of the inflammatory cytokine response to impact by curcumin and dexamethasone results in chondroprotection, further supporting the role of cytokines in chondrocyte death post impact and suggesting cytokine release modulation as a possible target for chondroprotection.

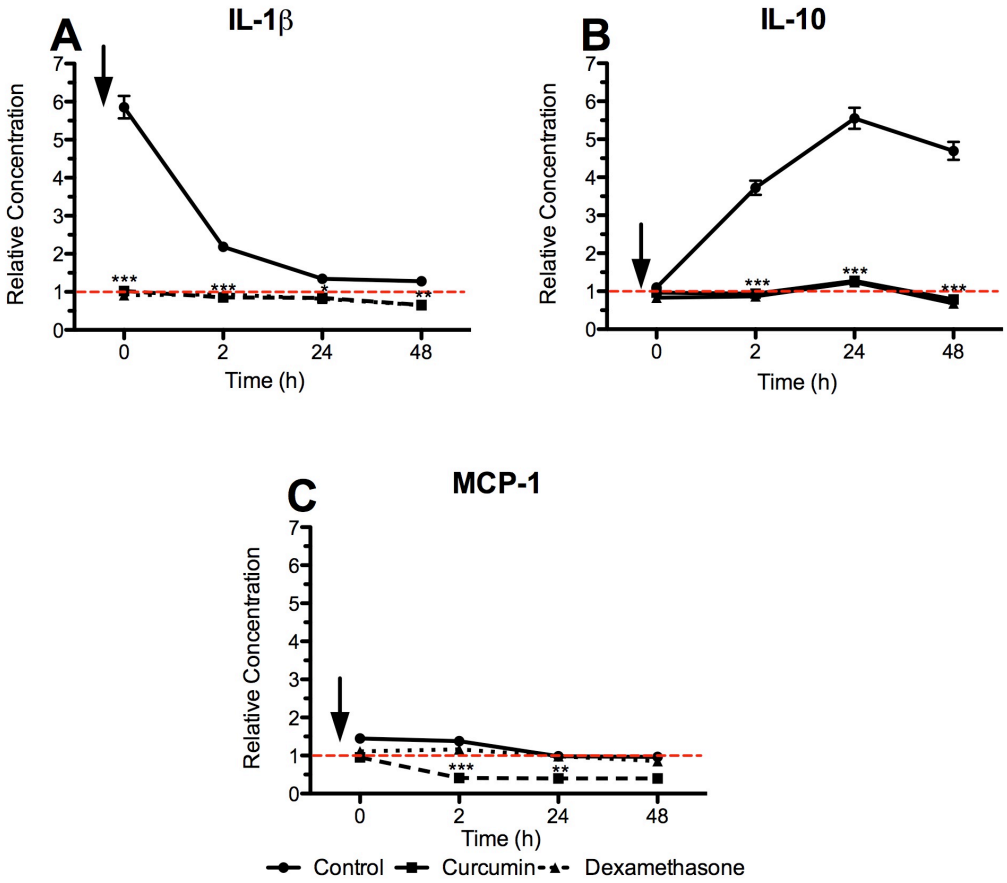
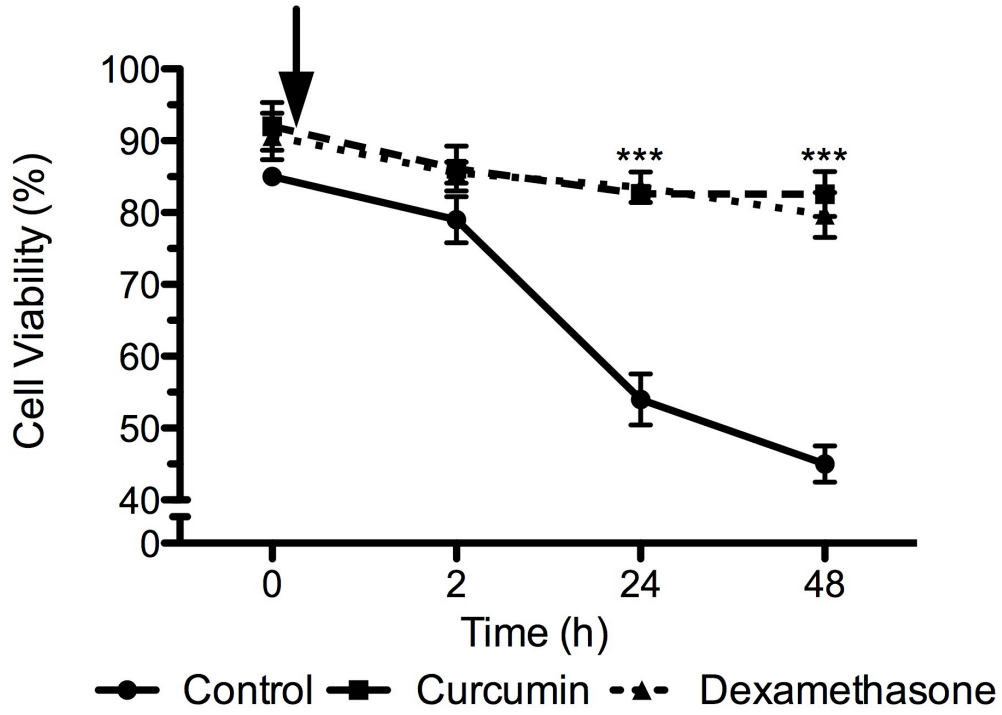


Figure 5.11: Effect of Curcumin and Dexamethasone on pro and anti-inflammatory cytokine release following post-mechanical trauma.

Cartilage explants were pre-treated for 1 h with control isotonic, curcumin (50 μM) or dexamethasone (1 μM) supplemented DMEM and subjected to a single mechanical insult. Supernatant were collected at 2, 24 and 48 h post mechanical trauma and analysed by ELISA for IL-1β;(Panel A), IL-10 (Panel B) and MCP-1(Panel C). Data is mean ± s.e.m (cytokine concentration relative to non-impacted control samples) from N=12 explants from 3 distinct experiments. Arrow denotes impact point.



Condition	Initial rate of chondrocyte death (%.h^{-1})	Subsequent rate of chondrocyte death (%.h^{-1})
Control	2.75 ± 0.14	0.66 ± 0.03
Curcumin	2.95 ± 0.01	0.07 ± 0.02 ***
Dexamethasone	2.52 ± 0.03	0.13 ± 0.09 ***

Figure 5.12: Curcumin and dexamethasone pre-treatment protects against cell death post mechanical impact.

*Cartilage explants were pre-treated for 1 h with control isotonic, curcumin (50 μM) or dexamethasone (1 μM) supplemented DMEM and subjected to a single mechanical impact. Samples were loaded with Calcein AM (5 μM) and PI (1 μM) and visualised using CLSM prior to and at 2 h, 24 h and 48 h post mechanical impact. Imaris spot analysis was used to quantify cell viability. Both curcumin and dexamethasone were seen to protect against cell death by significantly decreasing the subsequent rate of chondrocyte death. N=12 from 3 distinct experiments. *p<0.05, **p<0.01, ***p<0.005 all vs control. Arrow denotes impact point.*

5.3 Summary

The relationship between increased force and inflammatory cytokines was investigated to determine the potential role of cytokines within the response of cartilage to mechanical impact and the potential implications in the *in vivo* system. Firstly, analysis of joint forces revealed that shoes decreased all leg joint forces, confirming a protective role for footwear. Additionally, dominant and non-dominant maximal knee force when running barefoot was observed to be non-proportional, indicating leg dominance has a non-standardised effect on knee force. MDL knee force when running barefoot revealed positive correlations with both pro (IL-1β and MCP-1) and anti-inflammatory (IL-10) circulating cytokines, suggesting increased knee force may be associated with increased inflammation.

Analysis of strike patterns demonstrated an increased heelstrike gait was associated with an increased MDL knee force when running barefoot, but did not show any increase in either pro or anti-inflammatory circulating cytokines. An increased pronated gait was also observed to be associated with increased MDL knee force when running, but additionally correlated with increased pro and anti inflammatory cytokines. These data suggested that a heelstrike, pronated gait was optimal for increasing force, but a solely heelstrike gait was optimal for increasing force without increasing inflammation.

IL-1β, MCP-1 and IL-10 were all observed to increase post exercise, for both AM and PM exercise, with pro-inflammatory cytokines (IL-1β and MCP-1) peaking immediately post exercise and anti-inflammatory cytokines (IL-10) peaking at 2 h post exercise, again for both AM and PM exercise. Peak pro-inflammatory cytokine concentration was observed to be increased post AM exercise compared to PM. Conversely, peak anti-inflammatory cytokine concentration was not seen to be significantly different between groups.

Mononuclear phagocytes were observed to be involved in a cytokine paracrine loop with articular cartilage, driven by IL-1β, with mechanical

impact resulting in increased production of IL-1β, IL-6, IL-8, IL-10 and MCP-1 from articular cartilage which induced an increased production of IL-1β and MCP-1 from mononuclear phagocytes. This, in turn, led to an increase of IL-1β from articular cartilage. Given the cumulative nature of this paracrine loop, it is likely to be regulated by additional factors.

Supernatants containing cytokines released by isotonic and hypo-osmotic samples in response to impact resulted in chondrocyte death, conversely, supernatants from hyperosmotic samples, shown to produce decreased cytokine concentrations, did not induce chondrocyte death. These data suggested a role for inflammatory cytokines within chondrocyte death in response to impact. This was further supported by the observation that stimulation with IL-1β concentrations comparable to those released by articular chondrocytes in response to impact additionally induced chondrocyte death.

Curcumin and dexamethasone were confirmed to have suppressive actions on the inflammatory cytokine response to impact and further observed to protect against cell death upon mechanical impact by decreasing the subsequent rate of cell death. These data indicated a role for inflammatory cytokines in the second phase of chondrocyte death post mechanical impact, and further suggested cytokine release modulation as a possible chondroprotective mechanism.

Chapter 6: Discussion

Musculoskeletal conditions, such as osteoarthritis (OA), are the second largest cause of lost working days within the UK (after anxiety and depression), with ~10 million days lost in 2006/7 (ARC, 2008). Additionally, musculoskeletal conditions are the biggest contributor to the number of claimants for disability living allowance, representing more that 18 % (ARC, 2008). Combined, this brings an economic annual cost of £5.7 billion, solely within the UK (ARC, 2008). OA is the most common form of arthritis, affecting more than 8 million people within the UK and with an estimated 1 in 5 adults over the age of 50 suffering OA in one or both of their knees (ARC, 2008). Given the prevalence of OA and its economic and social impact, it is of no surprise that research into OA is an ever-growing field, but despite this plethora of research, the multifactorial nature of the disease has, as yet, impeded the discovery of a cure (Arthritis Research UK, 2010). However, there are a number of known risk factors for the development of OA, indicating possible areas of intervention, with age being the most prominent, and others including: (1) genetic predisposition, (2) obesity and (3) abnormal loading (Felson *et al*., 2000). Whilst age appears the largest risk factor, with the risk of OA increasing with age, it is noted that OA can occur in people of all ages including the young and those in mid-life, and additionally, that not all elderly develop OA. With obesity, another major risk factor for OA development, due to the excessive loading on the joints, it is suggested that the 'wear and tear' of joints over the years due to repetitive or excessive loading is a cause of OA (Arthritis Research UK, 2010).

When considering repetitive joint loading as a risk factor for OA, an obvious area of interest is high impact sports, including activities such as endurance running, sprinting, football and tennis (Lequesne *et al*., 1997). Despite this, there has been little to no research on the links between sport participation or sport injury and OA, until recently brought to light by the Arthritis Research UK campaign entitled "Taking the pain out of sport", spear-headed by its "Tackling osteoarthritis in sports" conference in October 2010 (Arthritis Research UK, 2010). Whilst it is acknowledged that joint injury through sport increases OA risk (Buckwalter, 2004, Lotz, 2010, Brown *et al*., 2006), evidence also suggests that the repetitive loading involved in high impact sports, such as endurance running, can also increase OA risk

(Spector *et al*., 1996, Marti *et al*., 1989). Indeed, animal models involving the application of loading stresses comparable to joint loading forces in sport have demonstrated the induction of OA (Andriacchi *et al*., 2009, Milentijevic *et al*., 2005). Based on this current evidence there is clearly a need for further research into the links between repetitive loading and OA, and the mechanisms involved. This would enable the provision of preventative advice for athletes and amateurs alike, targeting of high-risk groups, and elucidation of targets for chondroprotective, preventative strategies (Arthritis Research UK, 2010, Lequesne *et al*., 1997).

This study investigated the cellular responses of chondrocytes to mechanical impact, and the effects of possible chondroprotective agents. This would allow for a better understanding of the effects of loading on cartilage tissue with a view to identifying viable targets for preventative strategies in individuals exposed to high impact, repetitive loading.

Mechanical impact of 6.47 KPa and 1.14N was used to investigate the cellular viability responses of articular chondrocytes to impact. Whilst it is acknowledged these pressure values are lower than *in vivo* physiological values, approximately 5 MPa upon walking (Hodge *et al*., 1986, Urban, 1994), it is to be noted that within this model samples did not have the protection of the subchondral bone or synovial fluid that they are afforded *in vivo* (Jeffrey *et al*., 1995). The model used here is intended not to replicate *in vivo* pressures, nor study injurious, matrix damaging loads, but to provide a mechanical impact of sufficient magnitude to study cellular responses, and thus elucidate potential areas of interest for chondroprotection.

6.1 Mechanical Impact and Chondrocyte Viability

Mechanical impact has been previously shown to result in decreased chondrocyte viability and matrix deformation (Milentijevic and Torzilli, 2005, Szczodry *et al*., 2009). Here it was observed that mechanical impact induced a biphasic decrease in viable cells, with initial, localised chondrocyte death being observed within the first 2 hours post mechanical impact. This was subsequently followed by a less acute rate of death of 0.66±0.03 $\%.h^{-1}$ between 2 and 48 hours post mechanical impact (*Fig. 3.4*). The data reported here is in agreement with published findings showing that chondrocyte death occurs following mechanical impact (Chen *et al*., 2001, Amin *et al*., 2009, Bush and Hall, 2001b, Bush *et al*., 2005, D'Lima *et al*., 2001a, D'Lima *et al*., 2001b, Milentijevic *et al*., 2005, Milentijevic and Torzilli, 2005, Szczodry *et al*., 2009, Tew *et al*., 2000), as is the observation of biphasic death (Bush *et al*., 2005, Chen *et al*., 2001), although the time course of the phases and the mechanism of death are as yet inconclusive. This can no doubt be attributed to the variety of techniques used to apply load, including drop tower (Szczodry et al., 2009), cyclic indentation (Chen et al., 2001), and cyclic compression (Campbell et al., 2007), and to quantify viability, as well as to the variety in load magnitudes used. Studies have reported initial rapid death with 3 min of impact and more expansive death over the subsequent 25 min in *in situ* bovine cartilage, with the Superficial Zone (SZ) displaying the most sensitivity (Bush *et al*., 2005). Whilst the mechanism of chondrocyte death post mechanical impact is still poorly understood, there have been reports of chondrocyte apoptosis post mechanical loading, with z-Vad.fmk, a broad spectrum caspase inhibitor, markedly reducing cell death post a single static 14 MPa load in human articular cartilage, thus suggesting caspase-dependent apoptosis as the mechanism of cell death (D'Lima *et al*., 2001a). Conversely, there is also evidence for necrosis post mechanical loading, with no nuclear fragmentation, a marker of apoptosis, being observed post 6 hour cyclic loading or 3 hour static loading of up to 1 MPa (Lucchinetti *et al*., 2002).

To further investigate the mechanisms of chondrocytic death post mechanical impact, samples were subjected to mechanical impact and

cultured at 20°C and at 37°C to elucidate the role of active cell processes within chondrocyte death. Whilst it is known that apoptosis is an active cellular process, necrosis is considered a passive process, thus apoptosis should be impeded by decreased temperature, in contrast to necrosis (Duprez *et al*., 2009). Observed here, initial death post mechanical impact was not affected by the decreased temperature, providing further evidence for necrosis being the initial mechanism of chondrocyte death (*Fig. 3.10*). Conversely, subsequent death was impeded by the lower temperature, suggesting the mechanism of subsequent death requires active cellular processes and is thus likely to be apoptosis (Duprez *et al*., 2009).

It has been reported that ethylene glycol tetraacetic acid (EGTA) has prevented cellular apoptosis in chick chondrocytes when induced by inorganic phosphate (Mansfield *et al*., 2003), and similarly in murine macrophages, when apoptosis was induced by cadmium, a carcinogenic metal found in cigarette smoke (Kim and Sharma, 2004). Additionally, previous work in bovine *in situ* chondrocytes has shown that removal of $[Ca^{2+}]_0$ by EGTA significantly reduced superficial zone chondrocyte death post scalpel induced injury when compared to calcium-rich conditions (Amin *et al.*, 2009). To determine the role of extracellular calcium ($[Ca^{2+}]_0$) in chondrocyte death post mechanical impact, $[Ca^{2+}]_0$ was removed via EGTA. EGTA incubation was observed to decrease the subsequent rate of chondrocyte death post mechanical impact, but conversely not the initial rate, strongly suggesting the involvement of apoptosis in the subsequent phase of chondrocyte death post mechanical impact, but not the initial phase (*Fig. 3.12*). Calcium dependent apoptosis has been similarly observed post impact loading in equine articular cartilage, whereby increased intracellular calcium ($[Ca^{2+}]_i$) following single impact induced mitochondrial depolarisation 3-6 hours post impact leading to chondrocyte apoptosis. Sequestering of $[Ca^{2+}]_0$ by EGTA inhibited both the observed mitochondrial depolarisation and apoptosis (Huser and Davies, 2007).

The data presented here suggests a role for both necrosis and apoptosis within chondrocyte death post mechanical impact, with the initial phase likely to be necrosis resultant from chondrocyte injury at the point of impact,

and the subsequent phase to be spreading apoptosis. A similar observation of biphasic death has been previously observed over comparable time periods, whereby Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay, a DNA fragmentation detection assay, was implemented at 4 and 48 hours post loading, determining the percentage of death achieved through apoptosis post cyclic indenting loading in canine cartilage (Chen *et al*., 2001, Duprez *et al*., 2009). The study reported that initial death up to 4 hours was not determined to be apoptotic, thus indicating necrosis was observed initially. Conversely, cell death measured at 48 hours was confirmed as apoptosis.

This study thus concludes that mechanical impact induces biphasic chondrocyte death, as previously described, whereby initial necrotic death is followed by temperature and calcium dependent apoptosis.

6.2 The Link Between Chondrocyte Volume and Viability Post Mechanical Impact

Cartilage osmolarity changes dynamically in response to the ever-changing extracellular environment (Stockwell, 1979). Chondrocytes are constantly exposed to mechanical loading of a variety of pressures, depending on activity and posture, which alter the extracellular environment (Urban, 1994). Loading induces tissue deformation, leading to an increase in hydrostatic pressure and thus disturbance of the osmotic balance within the tissue (Urban, 1994). Chondrocytes, as most cell types, are known to be osmotically sensitive, whereby changes in extracellular osmolarity induce a volume response (Bush and Hall, 2001b, Hoffmann and Dunham, 1995, McGann *et al*., 1988). Indeed, despite the mesh network of the extracellular matrix (ECM) that surrounds the chondrocytes and thus could possibly restrict volume changes, it has previously been observed that chondrocytes behave as perfect osmometers, following the classic Boyle-van't Hoff relationship (Bush and Hall, 2001b). This observation is further confirmed by data presented here (*Fig. 6.1*), whereby chondrocyte volume changes are proportional to changes in osmolarity, further validating iso-surface volume analysis as a viable technique for determining *in situ* chondrocyte volume. Given that normal walking results in a fluid loss of 1-5 % (Weightman, 1979), and the diurnal cycle of activity and rest 20 % fluid loss in the intervertebral discs (Boos *et al*., 1993), it is evident that chondrocyte volume will fluctuate during the course of a day. Cell volume maintenance is imperative for effective cell function (Urban *et al*., 1993), evidenced by the observed cell swelling in degenerate chondrocytes in OA tissue (Bush and Hall, 2003). Considering the osmotic, and thus volume, changes that articular cartilage is subjected to throughout the day, and the importance of volume in maintaining a healthy tissue (Urban *et al*., 1993), it is of interest to investigate the involvement of cell volume in maintaining cell viability post mechanical impact.

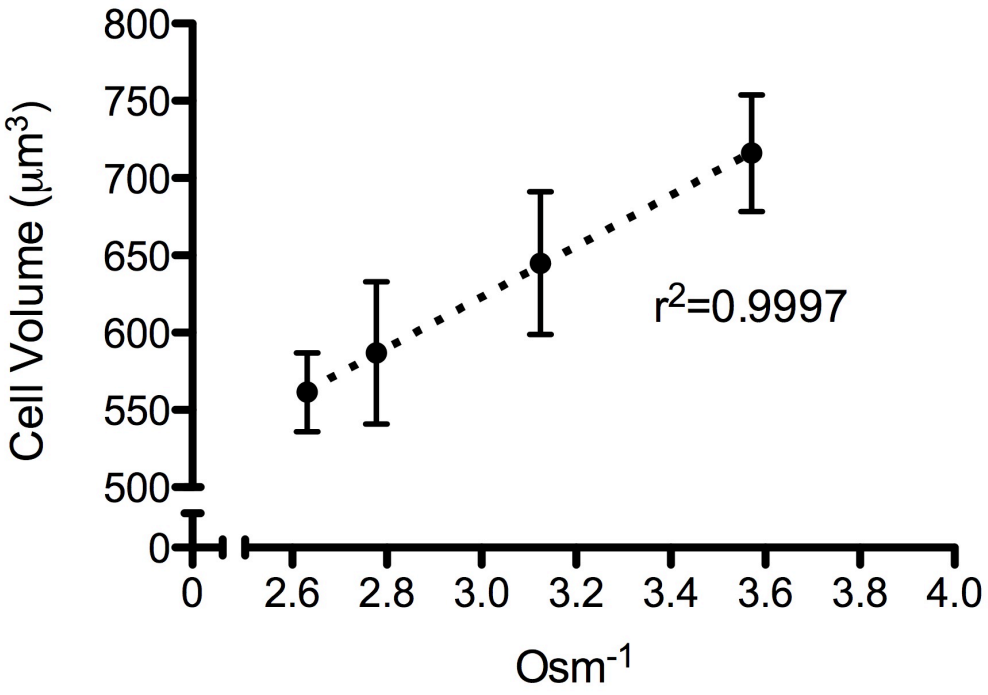


Figure 6.1: Osmotically induced changes in chondrocyte volume follow the Boyle Van't Hoff relationship

Mean experimental chondrocyte volume plotted against the reciprocal of the extracellular osmolarity following the Boyle van't Hoff relationship. The y-intercept was the osmotically inactive volume (130 μm^3), and the line of best fit for the data given had a regression coefficient (r^2 =0.9997) indicating that the chondrocytes behaved as perfect osmometers.

Articular cartilage explants were exposed to a hypo-osmotic (140 mOsm) or hyperosmotic (380 mOsm) challenge for 1 hour prior to mechanical loading, to induce cell swelling and cell shrinkage respectively. Initial chondrocyte volume was observed to inversely affect chondrocyte viability post mechanical loading, whereby increased volume due to cell swelling resulted in a decreased number of viable cells and, conversely, a decreased cell volume resulted in increased cell viability (*Fig. 3.4*). The data reported here supports previous studies demonstrating decreased sensitivity, in terms of cell viability, to mechanical loading in chondrocytes with decreased cell volume and similarly, increased chondrocyte volume resulted in increased cell death (Bush *et al*., 2005). Thus, whilst chondrocyte shrinkage can be termed chondroprotective, it is further evidenced here that chondrocyte swelling results in increased sensitivity to mechanical load, suggesting swollen OA chondrocytes (Bush and Hall, 2003) are more susceptible to damage and death following mechanical load.

Chondrocytes are known to respond to external stimuli, including osmotic changes, mechanical loading and fluid flow, by inducing mechanotransductory responses (Hall, 1998, Roberts *et al*., 2001, Yellowley *et al*., 1997), which have been shown to include changes in chondrocyte volume. Taking this into account with the data presented here, and supported by the literature (Bush *et al*., 2005), that has shown decreased cell volume to be chondroprotective, the question of whether chondrocytes have an endogenous chondroprotective mechanotransduction volume response to mechanical loading is raised. To investigate the presence of a possible chondroprotective volume response, chondrocyte volume was subsequently studied post mechanical impact in the presence and absence of hyperosmotic or hypo-osmotic conditions.

It was observed that chondrocyte volume decreased biphasically post mechanical impact, under isotonic, hyperosmotic and hypo-osmotic challenge (*Fig. 3.6*). This supports previous studies where decreased chondrocyte volume post mechanical load has been observed, although over shorter time periods (Bush *et al*., 2005). Interestingly, there are alternative reports of an increase in volume post injurious mechanical load

(Quinn *et al*., 1998). It is likely that these discrepancies are a result of non-standardised loading between studies, as an impact load great enough to induce physical injury to the tissue has been shown to disrupt the collagen network, induce proteoglycan (PG) swelling and thus induce reduced osmotic pressure and consequently cause cell swelling (Bush *et al*., 2005, Farquhar *et al*., 1996, Jeffrey *et al*., 1995). The data presented here is evidence of the cellular response to mechanical impact of pressure lower than that necessary to induce tissue injury, suggesting that chondrocytes display a biphasic impact-induced volume decrease (IIVD) upon non-injurious loading.

As apoptotic cells are observed to display a decrease in volume (Apoptotic Volume Decrease; AVD) prior to cell death (Bortner and Cidlowski, 2002), it should be considered whether IIVD is in fact AVD, and thus an artefact of chondrocyte death, or potentially a volume regulatory mechanism. Comparisons of cell viability post mechanical impact and IIVD reveal that samples subjected to a hyperosmotic challenge displayed no significant cell death (*Section 6.1*), however, these samples were still observed to undergo IIVD, suggesting no involvement for AVD. Closer inspection of the biphasic IIVD response in these chondroprotected samples revealed that although the cells still exhibit IIVD, a decrease in the subsequent rate was observed (*Fig. 3.6*). Taken together with previously discussed data suggesting a role for apoptosis in the secondary stage of cell death, it is possible that the secondary stage of IIVD is AVD. In addition, when impacted and cultured at a decreased temperature (20°C), whilst IIVD was still evident, the secondary phase was severely decreased (*Fig. 3.11*). As apoptosis is an active cell process (Duprez *et al*., 2009), this provides further evidence for AVD being responsible for the subsequent phase of IIVD and suggests a role for active processes in the mechanism of IIVD.

As the process of chondrocyte shrinkage shown here and previously (Bush *et al*., 2005) to be chondroprotective, elucidating the mechanism of the newly described IIVD is of interest. It is possible that mechanical loading is switching on a regulatory volume decrease (RVD) mechanism (Bush *et al*., 2005), whereby loading acts as a stimulus to induce RVD as a

chondroprotective mechanism. Whilst the mechanism of RVD within *in situ* chondrocytes is yet to be fully described, it is known to involve a transient rise in intracellular calcium ($[Ca^{2+}]_i$; (Hopewell and Urban, 2003, Urban *et al*., 1993) via gadolinium-sensitive stretch activated cation channels (SACC), gadolinium-insensitive calcium channels (Kerrigan and Hall, 2008) and intracellular store release (Sánchez *et al*., 2003) in isolated cells, in addition to an efflux of taurine, potassium (K^-) and chloride (Cl^-) ions via a volume sensitive organic anion channel (VSOAC)-like osmolyte channel (Hall, 1995). To investigate the potential of RVD mechanisms as part of the IIVD process, samples were incubated with REV 5901, a documented inhibitor of RVD (Bush and Hall, 2001a), or Tamoxifen, an inhibitor of VSOAC and thus RVD (Hall, 1995), prior to mechanical impact.

Tamoxifen incubated samples displayed an initial decrease in cell volume prior to mechanical impact, and subsequently conferred chondroprotection (*Fig. 4.14*). Interestingly, tamoxifen incubation abolished the IIVD response, indicating a potential role for VSOAC (Hall, 1995) within the mechanism of IIVD (*Fig. 4.16*). The chondroprotected response mediated by tamoxifen, despite the removal of IIVD, suggests that initial cell shrinkage is most important within chondroprotection, compared to the possible protection offered by IIVD. Additionally, REV 5901 induced a similar decrease in cell volume prior to mechanical impact, and subsequent chondroprotection (*Fig. 4.3*). However, IIVD was still observed, indicating IIVD to not be solely reliant on RVD mechanisms (*Fig. 4.5*).

Whilst REV 5901 is an inhibitor of RVD usually observed in response to hypo-osmotic challenge, recent report has suggested REV 5901 may induce RVD mechanisms under isotonic conditions (Qusous *et al*., 2011). Given the apparent role of calcium signalling in RVD (Kerrigan and Hall, 2008), a known calcium signalling pathway was investigated in REV 5901 samples. Articular cartilage explants were additionally incubated with rottlerin, an inhibitor of PKC (Gschwendt *et al*., 1994), U73122, an inhibitor of PLCβ_3 (Hou *et al*., 2004) or EGTA (Kerrigan and Hall, 2008). The addition of EGTA and thus the sequestering of $[Ca^{2+}]_0$ had no significant effect on the observed IIVD, suggesting IIVD is a $[Ca^{2+}]_0$ independent

mechanism (*Fig. 4.11*). Conversely, both rottlerin and U73122 eliminated the IIVD response observed, thus implicating the PKC/PLCβ_3 pathway in the mechanism of IIVD (Gschwendt *et al*., 1994, Hou *et al*., 2004). The PKC/PLCβ_3 pathway is known to induce IP_3 mediated intracellular Ca^{2+} store release, implying store Ca^{2+} release to be involved in the IIVD response, as opposed to $[Ca^{2+}]_0$ influx via SACC or other channels. Interestingly, studies have noted an increased sensitivity in IP_3 receptors and thus an increase in store-mediated $[Ca^{2+}]_i$ post mechanical loading (Zhang, 2006), further suggesting the involvement of PKC/PLCβ_3 pathway mediated store release in the chondrocyte response to impact.

This study observed a newly defined biphasic decrease in cell volume post mechanical impact, of which the initial phase was observed to be an active mechanotransduction mechanism, termed IIVD and the subsequent phase is likely to be AVD. IIVD was concluded to be dependant on PKC/PLCβ_3 pathway mediated intracellular Ca^{2+} store release and VSOAC channel activity (*Fig. 6.2*).

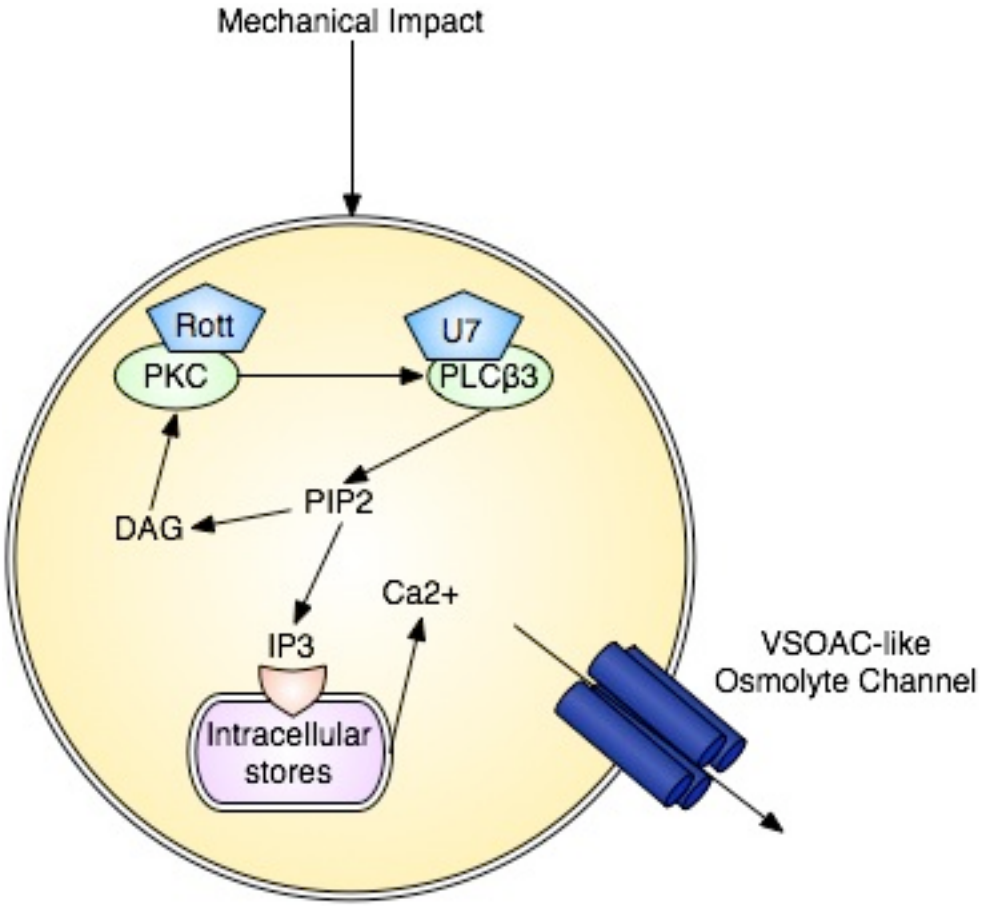


Figure 6.2: Theoretical model of the mechanism of IIVD

Mechanical impact induces signalling by the PKC/PLCβ_3 pathway, inhibited by rottlerin (Rott) and U73122 (U7) respectively, which activates intracellular store calcium release. Volume sensitive organic anion channel (VSOAC)-like osmolyte channel, possibly the same channel implicated in regulatory volume decrease (RVD) is additionally activated.

6.3 Filamentous Actin and Chondrocyte Viability Post Mechanical Impact

The actin cytoskeleton, comprising of filaments termed F-actin, is a dynamic network that cells maintain via a highly regulated balance of polymerisation and depolymerisation termed 'treadmilling' (Blain, 2009, Pollard and Borisy, 2003). Actin is involved in many cellular processes, including mechanotransduction, and most relevantly for this study, provides cells with mechanical integrity and thus the durability to withstand mechanical stresses (Blain, 2009, Grodzinsky *et al*., 2000, Wang *et al*., 1993). Given the importance of F-actin in maintaining a structurally sound cell, the effects of mechanical load on F-actin are of interest when studying the viability of chondrocytes. Thus, changes in F-actin were studied post mechanical impact, quantified independent of volume using CLSM and PQ.

Mechanical impact was observed to induce a rapid decrease in F-actin content, reaching a plateau at 2 hours post impact (*Fig. 3.7*), hereafter termed impact-induced actin decrease (IIAD). These data support previous reports of actin remodelling post impact, with a loss of actin fibres observed after application of hydrostatic pressure noted to be reversible after 2 h (Parkkinen *et al*., 1995). Interestingly, studies have observed a 20 fold increase in thymosin β_4 expression in *in situ* articular cartilage after dynamic loading (Blain *et al*., 2003). Thymosin β_4 is an actin sequestering protein which actively disassembles actin filaments by binding to globular actin monomers (G-actin) preventing polymerisation (Weber *et al*., 1992). Additionally, cyclic compression has been shown to upregulate cofilin, an actin disassembly protein, in isolated chondrocytes where a disassembly of actin was also observed (Campbell *et al*., 2007). The upregulation of actin depolymerising proteins thymosin β_4 and cofilin post mechanical load suggests a possible mechanism by which IIAD could be mediated.

The role of IIAD, and thus the importance of F-actin stability in chondrocyte viability post mechanical impact was further investigated using latrunculin B and jasplakinolide. Latrunculin B, purified from the Red Sea sponge *Latrunculia magnificans*, inhibits actin polymerisation and thus disrupts the

actin filaments (Spector *et al*., 1989) and jasplakinolide, a cyclic peptide isolated from the marine sponge *Jaspis johnstoni*, binds to and stabilizes F-actin (Bubb *et al*., 2000). Thus, latrunculin B and jasplakinolide was used as enhancer and an inhibitor of IIAD, respectively.

Latrunculin B was observed to give rise to a biphasic decrease in chondrocyte viability post mechanical impact, similar to that seen under isotonic conditions. Conversely, jasplakinolide induced chondroprotection, with no significant death observed post mechanical impact (*Fig. 3.14*). These data suggest a role for IIAD in chondrocyte death post mechanical impact, with prevention of IIAD being a potential target for chondroprotection. This is thought to be the first report of the effects of actin destabilisation on chondrocyte viability post mechanical loading, however a study using human alveolar epithelial cells observed latrunculin to enhance and jasplakinolide to have no effect on viability post single air bubble application (Yalcin *et al*., 2009).

Changes in osmotic environment have been previously shown to alter F-actin within chondrocytes whereby a decrease in extracellular osmolarity has been observed to decrease F-actin (Chao *et al*., 2006) and, similarly, hyperosmotic challenge to increase F-actin (Ong *et al*., 2010) in isolated chondrocytes. Here *in situ* chondrocytes were confirmed to have a similar F-actin response to extracellular osmolarity, with hypo-osmotic challenge reducing F-actin prior to mechanical impact, and hyperosmotic challenge increasing F-actin. Additionally observed here, for the first time, were the effects of osmotic challenge on IIAD, whereby both hypo-osmotic and hyperosmotic challenge were seen to display IIAD, but to a decreased rate when compared to isotonic controls (*Fig. 3.7*). It is hypothesised that the decrease in IIAD could be a result of the increased initial F-actin post hyperosmotic challenge and similarly, a result of the decreased initial F-actin post hypo-osmotic challenge. Interestingly, as IIAD is still observed under hyperosmotic challenge yet hyperosmotic challenge has chondroprotective effects, it is implied that increased initial F-actin prior to mechanical impact has a larger role in chondroprotection than prevention of IIAD.

Together these data suggest a role for rapid IIAD, possibly mediated by increased thymosin β_4 and cofilin post mechanical impact, in chondrocyte death, however, increased F-actin prior to mechanical impact appears to provide more effective chondroprotection than inhibiting IIAD using jasplakinolide.

6.4 The Role of Inflammatory Cytokines in Chondrocyte Viability Post Mechanical Impact

Whilst OA is classically considered not to be an inflammatory disease, there is a growing body of research supporting the role of inflammatory cytokines within the pathogenesis of OA (Pelletier *et al*., 2001). Increased concentrations of a range of pro and anti inflammatory cytokines have been observed in OA tissue, including IL-1β, TNF-α, IL-8 and IL-10 (Martel-Pelletier *et al*., 1999), although the exact role of these cytokines within the OA pathology is yet to be fully elucidated (Pelletier *et al*., 2001). It is known that articular cartilage is sensitive to inflammatory cytokines, whereby IL-1β and TNF-α are known to upregulate production of matrix metalloproteinases (MMPs) and inhibit collagen II synthesis, thus contributing to ECM degradation (Goldring *et al*., 1994, Goldring *et al*., 1988). In addition, IL-1β and TNF-α have positive feedback loops within the synovial joint, stimulating their production further as well as the production of other inflammatory cytokines including IL-6, IL-8 and nitric oxide (NO) (Pelletier *et al*., 2001).

A rise in inflammatory cytokines has been noted in response to differing exercise, with maximal exercise (Brenner *et al*., 1999, Suzuki *et al*., 2003a), resistance exercise (Brenner *et al*., 1999, Hirose *et al*., 2004, Nieman *et al*., 2004a, Smith *et al*., 2000), eccentric cycling (Toft *et al*., 2002) and endurance running (Brenner et al., 1999, Nieman et al., 2004b, Ostrowski et al., 1999b, Peters et al., 2001, Suzuki et al., 2000, Suzuki et al., 2003a, Suzuki et al., 2003b) all inducing cytokine changes. However, there has been limited work investigating the effects of exercise intensity or exercise force on cytokine production. Here, knee joint forces were quantified using forceplate collection and inverse dynamics, and compared with maximal inflammatory cytokine changes post submaximal exercise (60 % VO_2max). It was observed that increased knee force was positively correlated with increased production in serum inflammatory cytokines IL-1β, MCP-1 and IL-10, suggesting a role for exercise force magnitude on inflammatory cytokines (*Table 5.3*). This is was also observed in a previous study whereby high intensity exercise (>85 % VO_2max) induced an increased

plasma cytokine concentration compared to medium intensity exercise (60 % VO_2max) (Peake *et al*., 2005). Following this perceived role for exercise intensity in modulating cytokine concentration, it is thus of interest to consider factors which may influence exercise intensity, and thus joint impact, including running styles and protective footwear.

Investigated here were the effects of footwear, leg dominance and strike pattern on knee force. Footwear was, unsurprisingly, observed to have a suppressive, non-standardised, effect on knee force. Given the variety of footwear used, this effect was to be expected, as sports footwear is designed using a variety of different elastic materials to cushion the foot and absorb force (Nigg and Segesser, 1986). Leg dominance was additionally observed to have a non-standardised effect on knee force, whereby knee force was not habitually equal between legs, but the dominant leg did not have a uniform effect on force magnitude (*Table 5.2*). These data support previous studies observing ground reaction force (GRF) asymmetry between legs, unrelated to leg dominance in both young (23±4 years) and older (70±4 years) sprinters (Korhonen *et al*., 2010). Conversely, strike pattern exhibited a correlated effect on knee force whereby both a pronated gait and heelstrike were observed to increase knee force. Strike pattern is of interest in running injury, with two differing running styles currently adopted, 'pose' style forefoot and heelstrike running. Currently, there is no definitive conclusion of which style is superior in terms of force generation or injury prevention, although these data supports previous study that heelstrike increase lower limb force (Sol, 2001). Furthermore, whilst a pronated gait was observed to be associated with increased serum concentrations of IL-1β, MCP-1 and IL-10, heelstriking was not, despite both correlating with increased force (*Table 5.4/5*). The increase in inflammatory cytokine release in pronated gaits, could be an indicator of joint damage, as excessive pronation is linked to increased incidence of lower limb injury, including patellofemoral disorders (James B, 1978, Clanton, 1992, Hintermann and Nigg, 1998).

Further investigated here, were the cytokine changes post exercise, whereby serum cytokine concentration was analysed by ELISA up to 4

hours post submaximal (60 % VO_2max) exercise. Interestingly, whilst both IL-1β and MCP-1 were seen to peak immediately post exercise before resolving towards pre-exercise levels, IL-10 was observed to have a delayed onset, peaking at 2 hours post exercise before subsequently declining (*Fig. 5.3*). These findings are supported by previous studies which observed peak increases of pro-inflammatory cytokines, including IL-1β, immediately post marathon running (Ostrowski et al., 1999a). The delayed peak of IL-10 compared to the pro-inflammatory cytokine peak, similar to that observed in other reports (Cox *et al*., 2007), is thought to be a result of anti-inflammatory cytokine activity in response to pro-inflammatory cytokines, for the purpose of restricting further pro-inflammatory cytokine production (Peake *et al*., 2005, Toft *et al*., 2002).

In addition to contributing to OA pathology by inducing upregulation of MMPs and inhibiting collagen II synthesis (Goldring *et al*., 1994, Goldring *et al*., 1988), cytokines are additionally noted to contribute to OA pain by inducing hyperalgesia via a number of direct and indirect actions. IL1β is known to activate nociceptors directly via intracellular kinase activation, but it may also cause indirect nociceptor sensitisation (Sommer and Kress, 2004, Dray and Read, 2007). As OA pain is noted by sufferers to be more acute upon waking in the morning than in the afternoon and evening (Bellamy *et al*., 2002), it is suggested that inflammatory cytokines may exhibit a circadian rhythm. Considering the osmotically sensitive nature of the chondrocyte response to impact (*Section 6.2*), and the known involvement of inflammatory cytokines in ECM degradation in addition to cytokine involvement in morning OA pain, a possible diurnal inflammatory response to exercise is suggested.

The proposed diurnal inflammatory cytokine response to exercise was investigated whereby plasma cytokine concentrations were analysed for 4 hours post submaximal exercise (60 % VO_2max) commencing at 8 am or 6 pm. Both morning and evening exercise induced the same cytokine expression profile, where pro-inflammatory cytokines IL-1β and MCP-1 peaked immediately post exercise, and anti-inflammatory IL-10 peaked at 2 hours post exercise. However, it was noted that after morning exercise

peak IL-1β and MCP-1 concentration was double that of peak concentration post evening exercise. Conversely, IL-10 peak concentration did not show any significant differences between morning and evening exercise (*Fig. 5.3*). Whilst this is believed to be the first report of cytokine expression post diurnal exercise, previous studies have noted that cytokine production is differentially effected by serum cortisol, whereby cortisol suppresses pro-inflammatory cytokine production, thus pro-inflammatory cytokines, including IL-6 and IL-1β, exhibit a diurnal rhythm with peak levels during the night and early morning, at a time when plasma cortisol at its lowest (Cutolo and Masi, 2005).

It is important to note the *in vivo* inflammatory cytokine observations discussed are systemic cytokine responses to exercise and loading, whereby the cytokine changes observed are a product of the effects of exercise on the entirety of the body, thus it cannot be assumed that the localised cytokine response of articular cartilage is equal or indeed similar. Subsequently, the cytokine response to mechanical impact was here investigated *in situ*.

Chondrocytes are known to produce cytokines, including IL-1β, IL-8, IL-10 and MCP-1 in unstimulated conditions, as determined by antibody microarray of human isolated chondrocytes (De Ceuninck *et al*., 2004). Supernatants from non-impacted bovine samples were here analysed to determine basal rates of cytokines, which supported this report, demonstrating levels of IL-1β, IL-10 and MCP-1 measurable by ELISA (*Fig. 3.8*). IL-1β levels were comparable to those of MCP-1, however IL-10 concentrations were observed to be elevated, possibly a consequence of the dissection. Post mechanical impact, pro-inflammatory cytokines IL-1β and MCP-1 increased immediately following impact, before declining to near baseline levels. Anti-inflammatory cytokine IL-10 was additionally observed to increase immediately following mechanical impact, but continued to rise, peaking later at 24 hours post impact (*Fig. 3.9*). Observation of both pro-inflammatory and anti-inflammatory cytokines supports previous reports, whereby low grade mechanical load induces an anti-inflammatory response, and high grade load induces a pro-

inflammatory response, thought to be mediated via integrin signalling and the NF-κβ pathway (Deschner *et al*., 2003). These data suggests a pro-inflammatory cytokine response to high dynamic load, as modelled here, followed by an anti-inflammatory cytokine response restricting further pro-inflammatory cytokine production (Peake *et al*., 2005, Toft *et al*., 2002).

As normal walking is known to result in a fluid loss of 1-5 % (Weightman, 1979), and the diurnal cycle of activity and rest induces a 20 % fluid loss in the intervertebral discs (Boos *et al*., 1993), articular chondrocytes are likely to be of a decreased cell volume later in the day as a consequence of prolonged static loading. Conversely, OA chondrocytes are observed to be of increased cell volume, as a result of cell swelling (Bush and Hall, 2003). As previously noted (*Section 6.2*), changes in cell volume prior to mechanical load were observed to have significant effects on chondrocyte viability, and considering the potential role for inflammatory cytokines within the OA pathogenesis, the effects of osmotically induced chondrocyte volume changes on the inflammatory cytokine response are of interest.

Cytokine responses to mechanical impact were investigated prior to hyper and hypo-osmotic challenge. Both osmotic challenges were observed to produce the same cytokine response profile post mechanical impact as isotonic controls, whereby IL-1β and MCP-1 displayed an immediate increase following impact, and IL-10 peaked at 24 hours post impact. However, hypo-osmotic challenge induced an increased expression of all cytokines across the measured time course and, conversely, hyperosmotic induced a decreased expression (*Fig. 3.9*). Hypo-osmotic challenge has been often reported to induce an inflammatory cytokine response in many cell types, including rat hepatocytes (Kim *et al*., 2000) and human intestinal epithelial cells (Hubert *et al*., 2004), supporting the data collected here. However, hyperosmotic challenge is additionally reported to induce an increased inflammatory response in various cell types, including human limbal epithelial cells (Li *et al*., 2006), human mononuclear cells (Shapiro and Dinarello, 1997) and murine gastric epithelial cells (Zhang *et al*., 2006), contrary to data presented here. Despite these discrepancies, it should be noted that in these reports osmotic stress was the only challenge subjected

upon the cells, and thus the combination of hyperosmotic cell shrinkage followed by mechanical impact could have resulted in these differing reports. Additionally, the varying tissue and species could have had an impact. Interestingly, the *in vitro* cartilage cytokine response to mechanical load displayed a similar expression pattern to that of *in vivo*, whereby pro-inflammatory cytokines peaked immediately followed by a later onset anti-inflammatory cytokine peak. In addition, *in vivo* evening exercise resulted in decrease inflammatory cytokine production parallel to the lowered production seen in *in vitro* hyperosmotic conditions, a model of prolonged static loading.

Despite the correlation between the *in vitro* cartilage cytokine response and the *in vivo* systemic cytokine response, there are still multiple contributors to consider in the inflammatory cytokine response, solely within articular cartilage, including polymorphonuclear granulocytes (PMNs), mast cells and mononuclear phagocytes (Freemont, 1991), all of which are present within the synovial fluid. Mononuclear phagocytes have been observed to produce pro and anti-inflammatory cytokines, including IL-1β and IL-10, in addition to MMPs, have been identified in abundance within OA tissue (Benito *et al*., 2005, Farahat *et al*., 1993, Haywood *et al*., 2003) and additionally noted to have an increased number and function post exercise (Woods *et al*., 2000). Thus it is suggested that there is the potential for mononuclear phagocyte involvement in both OA pathogenesis and exercise induced inflammatory cytokine response. Synovial mononuclear phagocytes were isolated from bovine synovial fluid to investigate the potential role in cartilage inflammatory cytokine release post mechanical impact.

Synovial fluid derived mononuclear phagocyte supernatant was analysed by ELISA for baseline cytokine levels, which revealed transient release of IL-6 and IL-8, but primarily IL-1β, IL-10 and MCP-1, as previously reported (Bondeson *et al*., 2010). Supernatant containing cytokines produced by articular cartilage explants 4 hours post mechanical impact was used to stimulate mononuclear phagocytes to investigate the effect of impact-induced cytokines on mononuclear phagocyte expression, whereby both IL-

1β and MCP-1 production were observed to significantly increase (*Fig. 5.4)*. Subsequently, mononuclear phagocytes were co-cultured with impacted articular cartilage to investigate the summative effect of impact and stimulated mononuclear phagocytes, which revealed an additional increase in IL-1β release. Whilst it must be noted that these data are not a complete model for the inflammatory cytokine signalling within the synovial joint, as many other cells present within the synovial capsule are likely to be involved, these data newly describe a paracrine loop of cytokine release (*Fig. 6.3*). The paracrine loop is likely driven by IL-1β, inducing an amplified cytokine signal, similar to the previously reported paracrine loop between adipocytes and mononuclear phagocytes, driven mainly by TNF-α that aggravates inflammatory changes in obesity (Suganami *et al*., 2005).

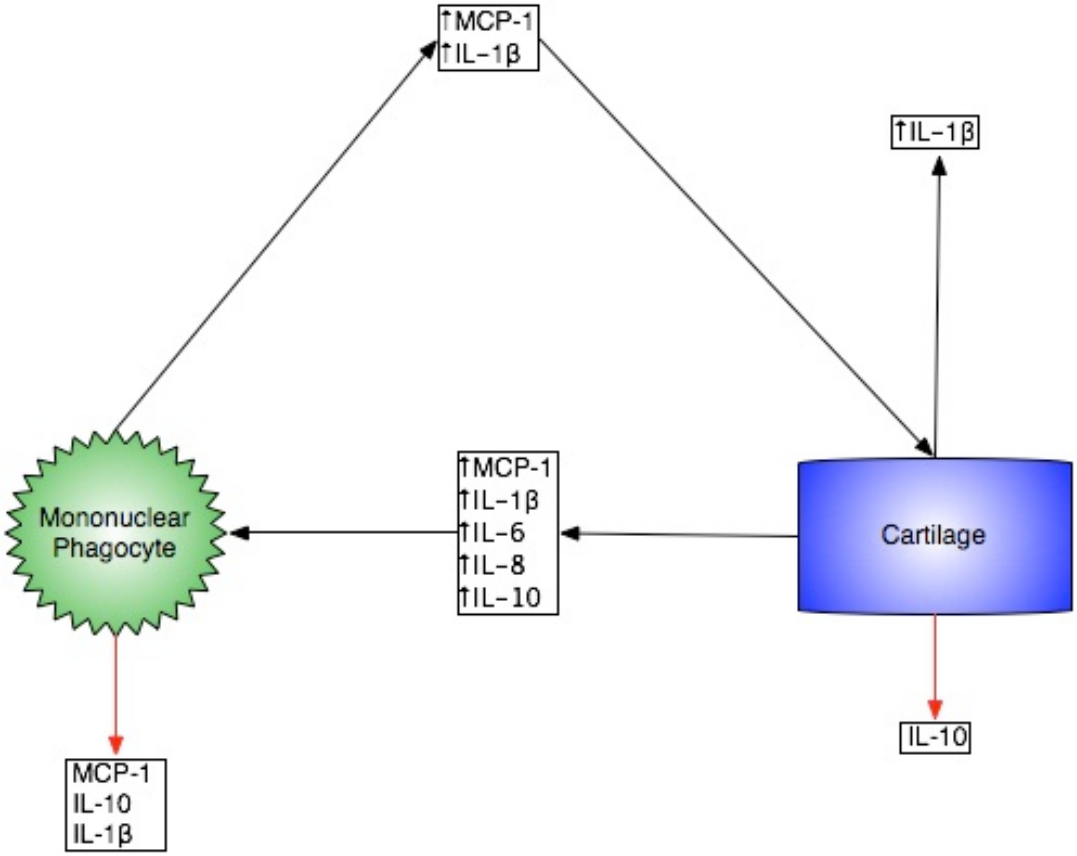


Figure 6.3: Model of the observed paracrine loop of cytokine release between articular cartilage and mononuclear phagocytes post mechanical insult.

Baseline levels of cytokines produced by articular cartilage and mononuclear phagocytes (black arrows) were observed to primarily be composed of IL-10 and MCP-1, IL-1β and IL-10 respectively. Single mechanical impact to articular cartilage increased levels of all measured cytokines which in turn increased concentration of MCP-1 and IL-1β from mononuclear phagocytes. Stimulation of articular cartilage with cytokines from these stimulated mononuclear phagocytes was observed to increase IL-1β concentration.

Given the reported milieu of inflammatory cytokines produced by articular chondrocytes in response to mechanical impact, and the known catabolic effects of cytokines on articular cartilage, the effects of these cytokines on apoptosis are of interest. Accordingly, supernatants from articular cartilage samples pre-incubated with isotonic, hyperosmotic or hypo-osmotic challenge, were removed post mechanical load, and subsequently used to stimulate non impacted cartilage samples. Chondrocyte viability, as determined by CLSM and spot analysis (*See Materials and Methods*) was observed to be significantly decreased in explants stimulated with supernatants from both isotonic and hypo-osmotic samples, but conversely, no significant viability change was observed in explants stimulated with supernatants from hyperosmotic samples (*Fig. 5.8*). As previously described here, hyperosmotic challenge induced a decrease in IL-β, MCP-1 and IL-10 expression compared to both isotonic and hypo-osmotic conditions, thus, supernatants containing increased levels of cytokines (isotonic and hypo-osmotic) were noted to induce chondrocyte death. Whilst cytokines have been reported to have a varied effect on cell death, depending on cell type and the cytokine (Haanen and Vermes, 1995, Yasuhara *et al*., 2005), IL-1β has been previously noted to induce cell death in chondrocyte-like ATDC5 cells by mitochondrial dysfunction (Yasuhara *et al*., 2005).

As IL-1β was found to be increased upon mechanical impact and a driver in the mononuclear phagocyte/cartilage paracrine loop, thus, the involvement of IL-1β in cytokine mediated chondrocyte death was investigated. The addition of IL-1β was noted to induce a significant decrease in chondrocyte viability, as measured by CLSM spot analysis, and an additional decrease in cell volume (*Fig. 5.6/7*). Similar studies have noted a decrease in chondrocyte viability when stimulated by recombinant IL-1β, whereby an Annexin-V-fluos staining kit was used to detect apoptotic cells, thus concluding that IL-1β induced chondrocyte death via apoptosis (Heraud *et al*., 2000). Furthermore, as apoptosis is known to involve a decrease in cell volume (AVD) (Bortner and Cidlowski, 2002), these data suggest IL-1β mediates chondrocyte death by apoptosis, and thus is likely to contribute to the secondary phase of death induced by mechanical impact (*Section 6.1*).

The described role for inflammatory cytokines within chondrocyte death post mechanical impact, and OA, suggests a possible area of interest in regards to chondroprotection, whereby anti-inflammatory agents may exhibit a chondroprotection response.

The chondroprotective potential of inhibiting inflammatory cytokines was investigated using curcumin, the active component of turmeric, which is known to have potent anti-inflammatory properties (Sharma *et al*., 2005), including down-regulation of MCP-1 expression (Wu *et al*., 2006, Herman *et al*., 2009) and dexamethasone, a potent anti-inflammatory drug used to treat inflammatory and autoimmune disorders including rheumatoid arthritis (Getting *et al.*, 2009). Curcumin and dexamethasone were confirmed to have anti-inflammatory effects, whereby a decrease in IL-1β to below baseline non-impact levels and a decrease in IL-10 to baseline non-impact levels was observed by both agents. Furthermore, dexamethasone decreased MCP-1 concentration to baseline non-impact levels, and curcumin to below baseline levels (*Fig. 5.8*). These data supported previous reports of inhibition of MCP-1 gene expression by dexamethasone (Ajuebor *et al.*, 1998). Curcumin and dexamethasone were noted to have no initial effect on chondrocyte viability, however, both agents exhibited a similar significant inhibitory effect on the secondary stage of chondrocyte death following mechanical impact, resulting in overall chondroprotection (*Fig. 5.9*). These data further support the conclusion that the secondary phase of impact induced chondrocyte death is mediated by cytokine induced apoptosis, as previously observed here (*Section 6.1/2*). Previous studies have made similar reports of the chondroprotective actions of curcumin, whereby curcumin treatment inhibited IL-1β induced apoptosis, via inhibition of activated NF-κβ translocation (Csaki *et al*., 2009). Conversely, previous work has reported dexamethasone to induce apoptosis within chondrocytic cell line HCS-2/8 with, however a concentration 25 x that used here (Chrysis *et al*., 2005), thereby allowing for these contradictory data, in addition to the possible differences between primary cell and cell line.

Together these data suggest that both *in vivo* exercise and *in vitro* mechanical load induce a release of inflammatory cytokines, with a decreased response in the evening or after prolonged static loading. Chondrocytes are noted to be sensitive to cytokines whereby they induce further cytokine production and mediate chondrocyte death by apoptosis, inhibited by anti-inflammatory agents. This suggests a role for both evening exercise and anti-inflammatory agents in chondroprotection.

6.5 A Role for Chondroprotective Agents

Loading is known to induce decreased chondrocyte viability and matrix deformation (Milentijevic and Torzilli, 2005, Szczodry *et al*., 2009) in addition to a rise in inflammatory cytokines (Brenner *et al*., 1999, Suzuki *et al*., 2003a), all of which are noted features of OA. Given the growing body of evidence that continued excessive mechanical loading can induce OA later in life (Arthritis Research UK, 2010), it is of interest to elucidate possible targets for intervention, whereby chondroprotective agents are employed in high risk groups prior to the onset of OA. It has been noted here that decreased cell volume and increased F-actin prior to impact in addition to inhibition of IIAD and inflammatory cytokines release all have chondroprotective effects.

6.5.1 Nutriceuticals

Many nutritional supplements (nutriceuticals) and complimentary therapies are utilised by OA suffers and by individuals hoping to maintain 'healthy joints'. Two of the most commonly used are chondroitin sulphate and glucosamine sulphate (ARC, 2009). Chondroitin sulphate constitutes the majority of glycosaminoglycan (GAGs) found within the ECM, branched from PGs. It has reported to stimulate cartilage repair, inhibit catabolic enzymes and have anti-inflammatory actions (Huskisson, 2008, Deal and Moskowitz, 1999). Orally administered chondroitin sulphate has been shown to be rapidly absorbed in the gastrointestinal tract, and a high content of chondroitin sulphate, labeled with the metastable radioactive tracer Technetium-99m, has been found in the synovial fluid and cartilage of both rats and humans (Ronca *et al*., 1998). Glucosamine sulphate is the principle substrate in the synthesis of PGs (Simanek *et al*., 2005), thus the theory behind its use is that it will provide increased potential for PG synthesis (Huskisson, 2008, Simanek *et al*., 2005, Pavelka *et al*., 2002). Glucosamine is confirmed to be increased in the synovial fluid of both OA patients and in an equine model following oral administration of glucosamine sulphate (Meulyzer *et al*., 2008, Persiani *et al*., 2007).

Both chondroitin and glucosamine sulphate were here observed to have chondroprotective actions, preventing any significant chondrocyte death post mechanical impact (*Fig. 4.21*). Additionally, both agents were observed to decrease chondrocyte volume prior to impact, a chondroprotective mechanism (*Section 6.2*) but still exhibited initial IIVD post mechanical impact (*Fig. 4.23*). This is the first report of chondroitin and glucosamine sulphate induced volume changes and thus the mechanism is not yet determined, however it is previously observed that glucosamine induces depletion of cellular ATP (Shikhman *et al*., 2009). Chondrocytes are known to express ATP dependent potassium (K_{ATP}) channels (Barrett-Jolley *et al*., 2010), whereby decreased intracellular ATP opens the channel, hyperpolarising the membrane and inducing Ca^{2+} influx via voltage-gated calcium channels (VGCCs) (Barrett-Jolley *et al*., 2010). VGCCs have been previously confirmed as active within freshly isolated bovine articular chondrocytes (Raizman *et al*., 2010). It is possible that glucosamine alters cell volume via this mechanism as changes in intracellular $Ca^{2+}$are known to be linked with volume. Similarly, chondroitin has been seen to alter cellular ATP in rat liver membranes (Vieira *et al*., 2001), thus could be acting via a similar mechanism. Additionally, whilst both chondroitin and glucosamine sulphate have been previously seen to activate PKC (Miki *et al*., 2002, Aoyama *et al*., 2005), blockage of PKC/PLβ_3 mediated intracellular calcium signalling using rottlerin (PKC inhibitor) or U73122 (PLβ_3 inhibitor) did not affect the pre-impact volume response, suggesting neither chondroitin or glucosamine sulphate initiated volume changes via this mechanism. Interestingly, removal of calcium by EGTA inhibited the decreased volume observed by both chondroitin and glucosamine sulphate, further supporting the proposed mechanism of volume decrease by ATP modulation and VGCC mediated influx. Rottlerin and U73122 did, however, abolish the observation of IIVD with chondroitin and glucosamine sulphate, further strengthening the role of PKC/PLβ_3 mediated store release in the mechanism of IIVD (*Section 6.2*).

Chondroitin and glucosamine sulphate were further observed to increase F-actin prior to mechanical impact, another chondroprotective response (*Section 6.3*), and additionally inhibited IIAD (*Fig. 4.24*). Neither of these

observations were affected by rottlerin, U73122 or EGTA, indicating the actions of chondroitin and glucosamine sulphate on F-actin are not mediated by extracellular Ca^{2+} influx or PKC/PLβ_3 mediated intracellular Ca^{2+} store release. No previous reports have been made regarding effects of glucosamine on actin, however it is noted that chondroitin stabilises actin in rats with induced acute necrotising pancreatitis (He and Guo, 2007). It can be suggested, that the reported alterations in cellular ATP by glucosamine (Shikhman *et al*., 2009) and chondroitin (Vieira *et al*., 2001) could induce the increase in initial F-actin and subsequent protection from IIAD by preventing actin depolymerisation. Whereby, ATP hydrolysis is required for actin filament 'treadmilling', the process of depolymerising and polymerising F-actin (Bugyi and Carlier, 2010).

Finally, both chondroitin and glucosamine sulphate decreased pro-inflammatory cytokines IL-1β and MCP-1 and induced the anti-inflammatory cytokine IL-10 following post mechanical impact to basal levels across the measured time course (*Fig. 4.25*). The observed anti-inflammatory effects of chondroitin sulphate are well documented (Bugyi and Carlier, 2010, Campo *et al*., 2009, Iovu *et al*., 2008, Martel-Pelletier *et al*., 2010), and noted to be driven by the inhibition of NF-$\kappa\beta$ activation (Iovu *et al*., 2008). Similarly, glucosamine sulphate is reported to inhibit NF-$\kappa\beta$ activation in OA chondrocytes (Largo *et al*., 2003) and in rat middle cerebral artery (Hwang *et al*., 2010). Although the data for these supplements looks promising as a protective agent for OA, the results generated here are in an *in vitro* model and further work is required to determine whether these observations translate to *in vivo* pre-clinical models of OA or patients.

6.5.2 RVD Inhibitors

Previously discussed inhibitors of RVD, REV 5901 and tamoxifen, were also noted to have chondroprotective effects, observed here for the first time (*Section 6.2*). In addition to the previously described chondroprotection and decrease in initial chondrocyte volume, both REV 5901 and tamoxifen induced an increase in F-actin prior to mechanical impact and subsequently protected against IIAD (*Fig. 4.6/17*). The effects of REV 5901 on actin, in addition to its effects on volume and viability, were abolished in the

presence of rottlerin or U73122 but not affected by EGTA. These data suggest a role for PKC/PLCβ_3 mediated Ca^{2+} store release, but not extracellular Ca^{2+} influx, in the mechanism of REV 5901, further supported by additional work from within the same research group using isolated chondrocytes (Qusous *et al*., 2011). Tamoxifen induced increases in F-actin have been previously noted in endometrial stromal cells (Albright *et al*., 1997), but conversely decreases in F-actin have been observed in lens epithelial cells (Verdugo-Gazdik *et al*., 2006). Tamoxifen is known to be anticalmodulin (Pawar *et al*., 2009) and could thus exhibit inhibitory effects on actin depolymerisation, whereby calmodulin is an upstream signalling molecule for the activation of cofilin, an actin-disassembly protein (Ono, 2007).

Furthermore, both REV 5901 and tamoxifen were observed to have anti-inflammatory actions, decreasing IL-1β, IL-10 and MCP-1 post mechanical impact to below baseline levels and maintaining them for the measured timecourse (*Fig. 4.8/18*). The anti-inflammatory actions of tamoxifen are supported by previous study whereby tamoxifen has been previously observed to be an inhibitor of NF-$\kappa\beta$ translocation, thus inhibiting further cytokine production (Richette et al., 2007). The actions of REV 5901 on modulating inflammatory cytokine concentration were not effected by rottlerin, U73122 or EGTA, indicating the anti-inflammatory actions of REV 5901 are the product of a separate mechanism of action.

REV 5901 is additionally an inhibitor of 5-lipoxygenase (5-LO) (Musser *et al*., 1987) which is an enzyme involved in the leukotriene pathway. The presence of 5-LO within cartilage tissue was confirmed here by LTB_4 assay, whereby LTB_4 is a product of the 5-LO pathway. LTB_4 was detected in non-impact samples and was additionally observed to be significantly increased post mechanical load. Whilst elevated LTB_4 has been previously observed within OA synovial fluid (Wittenberg *et al*., 1993), this is thought to be the first report of LTB_4 production within cartilage tissue. REV 5901 decreased LTB_4 to below non-impact levels (*Fig. 4.7*). Leukotrienes have been observed to induce production of cytokine synthesis, namely IL-1, in synovial cells (Kageyama *et al*., 1994), thus suggesting 5-LO inhibition

rather than PKC/PLCβ_3 as the mechanism of the anti-inflammatory actions of REV 5901.

6.5.3 What is the Most Efficient Chondroprotective Agent?

Together these data suggest chondroprotective actions for chondroitin and glucosamine sulphate; possibly via NF-$\kappa\beta$ translocation and ATP modulation, REV 5901; via 5-LO inhibition and PKC/PLCβ_3 mediated store release, and Tamoxifen; possibly via NF-$\kappa\beta$ translocation and inhibition of VSOAC and calmodulin. Additionally, the previously described anti-inflammatory agents, curcumin and dexamethasone, were confirmed to exhibit chondroprotective actions via modulation of inflammatory cytokine release (*Section 6.4*). Whilst chondroitin sulphate, glucosamine sulphate, tamoxifen and REV 5901 were observed to have similarly efficient chondroprotective actions, REV 5901 was the most efficient at increasing initial F-actin, but chondroitin sulphate and tamoxifen induced the greatest decrease in initial volume. Chondroitin sulphate and REV 5901 had the greatest impact on reducing inflammatory cytokine release (*Table 4.1*). Curcumin and dexamethasone were observed to have less chondroprotective effects than the other investigated agents, indicating that merely modulating inflammatory cytokine release is not enough for total chondroprotection. Thus, whilst REV 5901 was demonstrated to be the most proficient at the greatest number of chondroprotective actions, given the unknown safety of REV 5901, chondroitin sulphate is likely to be the most promising chondroprotective agent.

6.6 Conclusions

The cellular responses of chondrocytes to mechanical impact were here investigated to help elucidate possible targets for chondroprotection in high OA-risk individuals.

Mechanical impact was determined to induce biphasic chondrocyte death, characterised by an initial necrotic phase and a subsequent apoptotic phase. Additionally, biphasic decrease in cell volume post mechanical impact was observed, of which the initial phase was observed to be an active mechanism, termed Impact-Induced Volume Decrease (IIVD), and the subsequent phase to be Apoptotic Volume Decrease (AVD). The newly defined IIVD was concluded to be dependent on PKC/PLCβ_3 pathway mediated intracellular Ca^{2+} store release and VSOAC channel activity. Furthermore, mechanical impact was observed to induce a rapid decrease in F-actin, termed Impact-Induced Actin Decrease (IIAD; *Fig. 6.4*).

Both *in vivo* exercise and *in vitro* mechanical load induce a release of inflammatory cytokines, with *in vivo* concentrations being affected by joint magnitude and strike patterns. Load induced inflammatory cytokine increase was determined to be decreased in the evening or after prolonged static loading.

Chondroprotective actions were determined to be induced by decreasing pre-impact cell volume, increasing pre-impact actin and inhibiting IIAD. Additionally, inflammatory cytokines were noted to mediate chondrocyte death by apoptosis, thus anti-inflammatory actions were also deemed chondroprotective.

Nutriceuticals, chondroitin and glucosamine sulphate were determined to have chondroprotective actions, possibly via NF-$\kappa\beta$ translocation and ATP modulation. Similarly RVD inhibitors REV 5901 and Tamoxifen exhibited chondroprotective mechanism, via 5-LO inhibition and PKC/PLCβ_3 mediated store release, and NF-$\kappa\beta$ translocation and inhibition of VSOAC and calmodulin respectively. Anti-inflammatory agents, curcumin and

dexamethasone had less effective chondroprotective actions, via inhibition of inflammatory cytokines and thus apoptosis.

To conclude, mechanical impact induces changes to chondrocyte volume, F-actin and cytokine release, resulting in a decrease in chondrocyte viability (*Fig. 6.4*). Cartilage was revealed to have a 'diurnal' response to impact, whereby 'PM' cartilage, as modelled by hyperosmotic challenge, was determined to be chondroprotective. Similarly, investigation of diurnal exercise revealed PM exercise to induce a less severe inflammatory cytokine response, thus high impact exercise is recommended to be carried out in the evening rather than the morning. Exploration of possible chondroprotective agents revealed that there is a place for chondroprotection in high-risk groups, providing intervention occurs before OA onset, with current nutriceuticals and natural anti-inflammatory agents proving effective *in vitro*, and chondroitin sulphate likely to be the most promising.

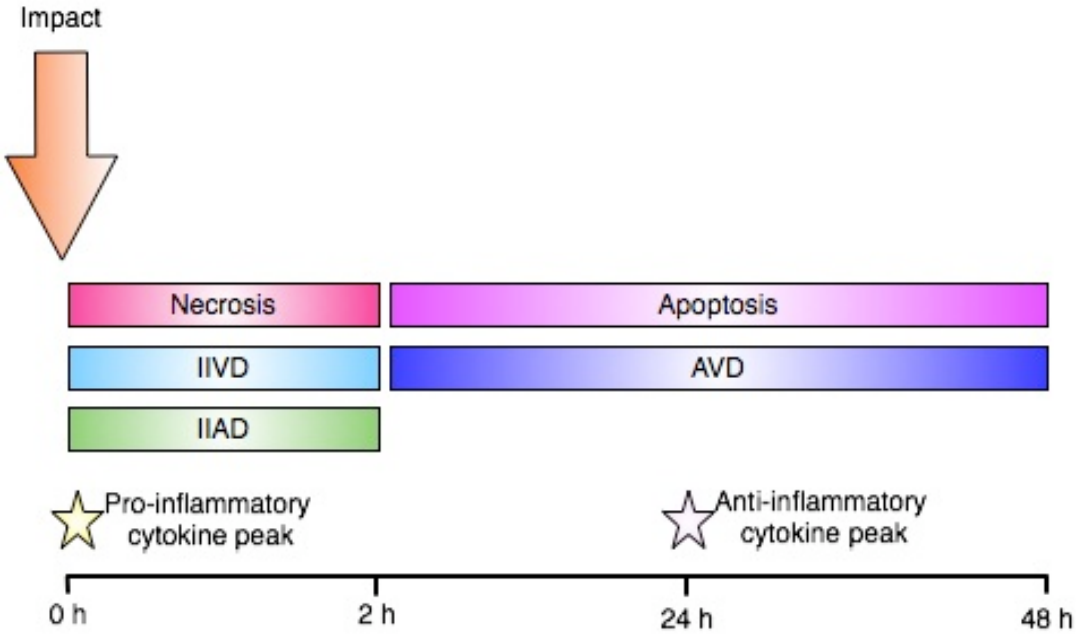


Figure 6.4: Summary of the responses of articular chondrocytes to mechanical impact.

Impact induces a biphasic decrease in chondrocyte volume, a rapid initial phase and a subsequent slower phased linked to apoptosis. Additionally, impact induces a rapid F-actin decrease and increased pro- and anti-inflammatory cytokine release, whereby pro-inflammatory cytokines peak immediately post impact and anti-inflammatory cytokines peak at 24 h. These responses resulted in biphasic chondrocyte death, with the initial rapid phase likely to be necrosis and the slower subsequent phase to be apoptosis. IIVD: Impact Induced Volume Decrease, AVD: Apoptotic Volume Decrease, IIAD: Impact Induced Actin Decrease.

6.7 Future Work

Future work to support these data includes further research into the mechanisms involved in IIVD. As the PKC/PLCβ_3 $[Ca^{2+}]_i$ store release signalling pathway was reported here to be involved, $[Ca^{2+}]_i$ levels post impact could be determined using CLSM, followed by investigation of the magnitude of the IIVD response following emptying of intracellular stores by thapsigargin. Additionally, the contribution of known membrane channels would be investigated by selective inhibition, including inhibition of SACC by gadolinium and VGCC by nimodipine. The biphasic cell death post impact would be further investigated to confirm necrosis and apoptosis via TUNEL technique and the zonal viability response to impact investigated by transverse sectioning of articular cartilage explants.

Further investigation into the cytoskeletal responses to impact would involve the utilisation of CLSM techniques and antibody labelling to investigate the responses of tubulin and vimentin to impact. Additionally, PCR and western blots would be used to determine changes in expression of cytoskeletal regulatory proteins, such as cofilin, profilin, gelsolin and thymosin β_4, post impact on both an mRNA and protein level respectively. Thus furthering investigation of the mechanism behind IIAD.

An *in vivo* animal model would be developed for further investigation into the chondroprotective potential of chondroitin sulphate, whereby the effects on articular cartilage integrity following repetitive high impact exercise of chondroitin sulphate administration would be analysed. Subsequently, a long-term *in vivo* human randomised controlled trial of chondroitin sulphate administration in high risk individuals would be developed, whereby chondroitin efficacy would be analysed by joint MRI, serum cytokine levels and injury/pain monitoring by questionnaire.

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Appendices

Appendix I: Training and Injury Questionnaire

MEDICAL QUESTIONNAIRE

This form is used as a pre-participation health and risk factor screening device and should be completed **prior to the commencement of an exercise test**.

The information obtained in this medical assessment will be kept as **CONFIDENTIAL**. Only the staff member related to the exercise test may access to the information.

Surname (Mr., Mrs., Ms.): _____

Given Names: _____

Date of Birth:
_____

Address:
_____

_____ Postcode:
_____

Contact Telephone: _____ (Home) _____ (Work)

FAMILY MEDICAL HISTORY.
Has any near relative brother (B), sister (S), father (F), mother (M), grandparents (GP) suffered:

Please tick the appropriate column

	No	Yes	Relation	Age	Remarks /Details
Apoplexy (stroke)					
Congenital heart trouble					
Rheumatic heart disease					
Heart operation					
Angina					
Heart attack					

Sudden death					
High blood pressure					
High cholesterol					
'Hardening of arteries'					
Asthma					
Lung disorder					
Bronchitis, emphysema					
Hay fever					
Diabetes					
Gout					
Arthritis					
Epilepsy					

PAST MEDICAL HISTORY

Have you suffered any of the following conditions at any time?
(Please tick the appropriate column)

	No	Yes	Details
Rheumatic or scarlet fever			
Heart trouble or murmur			
Heart palpitation			
High blood pressure			
Heart attack			
Chest pain/Angina			
Stroke			
Disease of arteries or veins			
Undue limiting shortness of breath with exercise			
Fainting or blackout			
Loss of consciousness or fainting with exercise			
Epilepsy			
Lung or bronchial disease			
Asthma			
Hay fever			
Anaemia			

Diabetes			
Thyroid disease			
Arthritis, rheumatism or gout spondylitis, disc trouble or back injury			
Serious accident or injury			
Surgical operation			
Congenital abnormality			
Other serious illness (or conditions that may affect exercise)			
For female only: Having normal/regular periods			

CURRENT MEDICATION

State the name and dosage of any drugs or medicines that you are taking regularly:

Drug	Dose	Time of last dose

Signed: _____ Name: _____

Date: _____

TRAINING & INJURY QUESTIONNAIRE

Name:.....

Club Name/ Region: .....

Date of Birth.....

Age.....Mass (Kg) :..... ..... Height (m):.....

CONTACT INFORMATION

Address:.....

.....

.....Postcode.....

.....

Telephone

Number):.....

E-

mail.....

PERSONAL BEST TIMES

Sport.....

Level reached (club/county etc).....

Personal best time/distance etc.....

CURRENT TRAINING

Please complete the below form for an average 2 weeks. Please include as much detail as possible.

		AM	PM
Week 1	Monday		
	Tuesday		
	Wednesday		
	Thursday		
	Friday		

	Saturday		
	Sunday		
Week 2	Monday		
	Tuesday		
	Wednesday		
	Thursday		
	Friday		
	Saturday		
	Sunday		

TRAUMATIC/OVERUSE INJURIES WITHIN THE PREVIOUS YEAR

(an injury is defined as any musculoskeletal problem that caused you to stop training for at least one day, reduce mileage, take medicine or seek medical aid).

Have you had any traumatic/overuse injuries within the past five years? Ring answer Yes No

Please give details of traumatic injury/ injuries, including date:

i Neck .....
ii Upper Back .....
iii Hamstrings .....
iv Calf .....
v Achilles Tendon.....
vi Combination.....
vii Shoulder .....
viii Lower Back.....
ix Anterior Thigh.....
x Knees.....
xi Ankles.....
xii Other (state what).....

Are you injury- free at the moment? Yes No

Is there any reason why you should not undergo physiological testing? Yes No

Signed: _____ Name: _____

Date: _____

Appendix II: Conference abstracts

THE EFFECTS OF REV5901 ON CHONDROCYTE VIABILITY FOLLOWING MECHANICAL TRAUMA

E. Parker, S.J. Getting, F. Hucklebridge, V.E. Vleck & M.J.P. Kerrigan

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Mechanical trauma through repetitive, high-impact sport contributes to the onset of osteoarthritis. Hypertonicity protects chondrocytes from mechanical trauma[1] suggesting a role for cell volume in this process. Since the 5-lipoxygenase inhibitor REV5901 inhibits chondrocyte volume regulation, this study was designed to investigate whether this compound exerts a chondro-protective effect following mechanical trauma.

Cartilage explants were dissected from the joints of 18-21 month old steers into DMEM. Explants were incubated for 1h in either: isotonic (280mOsm), hypertonic (380 mOsm), DMSO supplemented (5μl/ml) or 50μM REV5901 DMEM and subsequently subjected to a single impact as previously described[2]. Chondrocyte viability was determined by confocal microscopy[2] and data expressed as mean ± s.e.m; *$p<0.05$. n = 5 distinct experiments.

Explants in isotonic or DMSO supplemented DMEM exhibited a decrease in cell viability from 79±0.05% & 83±1.63% to 45±2.52% & 50±3.45% at 48h post impact; *$p<0.05$ and *$p<0.05$ respectively. Conversely, when incubated with hypertonic DMEM or 50μM REV5901 there was no decrease ($p>0.05$) in chondrocyte viability from 89±3.65% to 90±4.51% to 93±2.63% to 86±3.34% at 48h.

These data suggest that REV5901 exhibits a chondro-protective effect in an *in vitro* model of mechanical trauma.

1. Bush, P., *et al*., Osteoarthritis and Cartilage, 2005. 13(1): p. 54-65.
2. From the University of Brighton, Winter 2008 Meeting: Proceedings of the British Pharmacological Society at http://www.pa2online.org/abstracts

THE EFFECTS OF REV5901 ON CHONDROCYTE VIABILITY FOLLOWING MECHANICAL TRAUMA

E. Parker, S.J. Getting, F. Hucklebridge, V.E. Vleck & M.J.P. Kerrigan

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Cartilage explants were dissected from the joints of 18-21 month old steers into DMEM. Explants were incubated for 1h in either: isotonic (280mOsm), hypertonic (380 mOsm), DMSO supplemented (5μl/ml) or 50μM REV 5901 DMEM and subsequently subjected to a single impact as previously described[2]. Chondrocyte viability was determined by confocal microscopy[2] and data expressed as mean ± s.e.m; *$p<0.05$. n = 5 distinct experiments.

Explants in isotonic or DMSO supplemented DMEM exhibited a decrease in cell viability from 79.0 ± 0.1% and 83.0 ± 1.6% for isotonic and to 45.0 ± 2.5% and 50.0±3.5% for DMSO supplemented following 48h post impact; *$p<0.05$ and *$p<0.05$ respectively. Conversely, when incubated with hypertonic DMEM there was no decrease ($p>0.05$) in chondrocyte viability (89.0 ± 3.7% to 90.0 ±4.5%) whilst incubation with 50μM REV 5901 did not alter cell vialbility (93±2.63% to 86±3.34%) at 48h. Pre-incubation with 50 μM REV 5901 resulted in a sustained decrease in cell volume ($p<0.01$) from 1547 ± 61 μm^3 to 1011 ±19 μm^3, which was not significantly different ($p>0.05$) when compared to cells in hypertonic DMEM.

These data suggest that REV5901 exhibits a chondro-protective effect in an *in vitro* model of mechanical trauma by causing a decrease in cell volume potentially *via* activating a volume-regulatory pathway.

1. Bush, P., *et al*., Osteoarthritis and Cartilage, 2005. 13(1): p. 54-65.
2. From the University of Brighton, Winter 2008 Meeting: Proceedings of the British Pharmacological Society at http://www.pa2online.org/abstracts/Vol6Issue4abst148P.pdf.

REV5901: Chondro-protective Effects Post Mechanical Trauma.

E. Parker, S. Noureen, S.J. Getting, V.E. Vleck, F. Hucklebridge & M.J.P. Kerrigan

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Mechanical trauma through repetitive, high-impact sport contributes to the onset of osteoarthritis. Hypertonicity protects chondrocytes from mechanical trauma[1] suggesting a role for cell volume in this process. Since the 5-lipoxygenase inhibitor REV5901 inhibits chondrocyte volume regulation, this study was designed to investigate whether this compound exerts a chondro-protective effect following mechanical trauma.

Cartilage explants were dissected from the joints of 18-21 month old steers (obtained with ethical permission) into DMEM. Explants were incubated for 1h in either: isotonic (280mOsm), hypertonic (380 mOsm), DMSO supplemented (5μl/ml) or 50μM REV5901 DMEM and subsequently subjected to a single impact as previously described (Bush *et al.,* 2005). Chondrocyte viability, volume and relative F-actin concentration were determined by confocal microscopy and data expressed as mean ± s.e.m; (Student T-test: $p<0.05$), n=45 cells each from 5 distinct experiments. Supernatant from the explants was analysed for inflammatory cytokines by ELISA at 0h, 2h, 4h, 24h and 48h post mechanical impact.

Explants in isotonic or DMSO supplemented DMEM exhibited a decrease in cell viability from 79±0.05% & 83±1.63% to 45±2.52% & 50±3.45% at 48h post impact; $p<0.05$ and $p<0.05$ respectively. Conversely, when incubated with hypertonic DMEM or 50μM REV5901 there was no decrease in chondrocyte viability from 89±3.65% to 90±4.51% to 93±2.63% to 86±3.34% at 48h. REV5901 incubated samples displayed a decrease in cell volume ($p<0.01$) from 1547±61μm^3 to 1011±19μm^3 2h post mechanical trauma when compared to control conditions. Additionally pre-incubation with REV5901 resulted in an increase ($p<0.05$) in relative F-actin (208.70±4.85AU) when compared to non-treated samples (185.00±6.36AU) prior to mechanical trauma and a significantly less acute decrease post trauma (REV5901 171.00±5.76AU; Isotonic 64.69±3.16AU both at 48h post trauma). Cytokine changes relative to control were significantly ($p<0.05$) decreased post trauma in samples pre-incubated with REV5901 (IL-1β: Isotonic 1.30±0.18pg/ml/g REV5901 0.55±0.21pg/ml/g; IL-10: Isotonic 1.18±0.09pg/ml/g REV5901 0.90±0.11pg/ml/g; MCP-1: Isotonic 1.46±0.20pg/ml/g REV5901 0.60±0.07pg/ml/g; all at 2h post trauma).

These data suggest that REV5901 exhibits a chondro-protective effect in an *in vitro* model of mechanical trauma by reducing cell volume and decreasing the release of inflammatory cytokines.

Bush, P., *et al*., Osteoarthritis and Cartilage, 2005. 13(1): p. 54-65.

Knee Forces And Inflammation: The Effects of Foot Strike Patterns And Footwear.

E. Parker, S.Domah, S.J. Getting, V.E. Vleck, F. Hucklebridge & M.J.P. Kerrigan

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With elite level athletes participating more frequently in their chosen sports, they are at an ever-increasing risk of injury. The long-term risk is the likely development of Osteoarthritis (OA). Joint loading frequency and magnitude, as well as build up of inflammatory cytokines, are both contributing factors to this condition. With many different training shoes on the market and differing running styles favored it is of benefit to investigate the affects of foot strike patterns and footwear on joint force magnitude and inflammation.

14 healthy male subjects aged 20-39 underwent anthropometric tests and were subsequently analysed for force magnitude and gait whilst running at 11 kmh^{-1}using a force mat, both barefooted and wearing documented footwear. Venous blood samples were taken at 0h, 2h and 4h post 30min treadmill run at 60% VO_2max. A control sample pre-exercise was also taken and inflammatory cytokine concentration determined by ELISA.

Results showed that whilst there was no correlation between knee force magnitude when barefoot and when in shoes (Pearson Coefficient = -0.41, $p>0.05$), there was a non-significant correlation between sole depth and maximal knee force (Pearson Coefficient = -0.56, $p>0.05$), suggesting other parameters of the footwear have an effect on force. Similarly no significant correlation was seen between dominant and non-dominant maximal knee force (Pearson Coefficient = 0.73, $p>0.05$), foot length and maximal knee force (Pearson Coefficient = 0.60, $p>0.05$), and percentage body fat and maximal knee force (Pearson Coefficient = 0.66, $p>0.05$). Conversely a significant correlation was displayed between maximal dominant barefoot knee force and relative maximal inflammation post exercise (IL-1β: Pearson Coefficient = 0.99, $p<0.005$, IL-10: Pearson Coefficient = 0.91, $p<0.05$, MCP-1: Pearson Coefficient = 0.89, $p<0.05$), suggesting increased joint forces result in elevations in pro-inflammatory cytokines. Additionally a high correlation was exhibited between both heel:ball (H:B) ratio and pronate:supernate (P:S) ratio with maximal dominant barefoot knee force (H:B; Pearson Coefficient = -0.89, $p<0.01$, P:S; Pearson Coefficient = -0.83, $p<0.05$) and with relative maximal inflammation post exercise (IL-1β: Pearson Coefficient = 0.96, $p<0.001$, IL-10: Pearson Coefficient = 0.90, $p<0.005$, MCP-1: Pearson Coefficient = 0.86, $p<0.05$), suggesting a increased heel strike gait, and increased pronation, both result in an increased joint force, and increased inflammation.

These data suggests that whilst it is clear footwear has an effect on joint forces, further investigation is needed into footwear. Additionally, it was concluded that a heel-strike, pronated gait is optimal for maximal joint force output, however it also increases the risk of OA development by increasing inflammation.

Chondroitin And Glucosamine Supplementation Results In Increased Chondrocyte Viability Post Mechanical Trauma *In Vitro*.

E. Parker, N. Begum, C. Wunderlich, N. Ravichandran, S.J. Getting, V.E. Vleck, F. Hucklebridge & M.J.P. Kerrigan

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Dietary supplements are increasingly being used for the symptomatic relief of osteoarthritis (OA). As supplements are not regulated as drugs, there is a lack of scientific support for their perceived benefits, and recent studies into their effects on articular cartilage have been conflicting. The use of supplements is becoming more widespread, thus it is in public interest to elucidate their mechanisms and effects. Subsequently this study investigates the effectiveness of two commonly used joint supplements (chondroitin sulphate; Ch and glucosamine sulphate; Gl) in chondro-protection post-mechanical trauma.

Cartilage explants were dissected from the joints of 18-21 month old steers (obtained with ethical permission) into DMEM. Explants were incubated for 1h in either: isotonic (280mOsm), Ch (0.3mg/ml) or Gl supplemented (0.1mg/ml) DMEM and subsequently subjected to a single impact. Chondrocyte viability, volume and relative F-actin concentration were determined by confocal microscopy and data expressed as mean±s.e.m; (Student T-test: $p<0.05$), n=45 cells each from 5 distinct experiments. Supernatants were analysed for IL-1β and MCP1 by ELISA at 0, 2, 4, 24 and 48h post mechanical impact.

Explants in isotonic DMEM exhibited a decrease in cell viability from 79±0.05% to 56.88±3.45% at 48h post impact ($p<0.01$). Conversely, when treated with Ch there was no decrease in chondrocyte viability from 99.08±3.65% to 98.71±4.51% at 48h. Additionally, samples treated with Gl exhibited a minor decrease in viability from 96.66±3.69% to 82.84±4.51% at 48h, which was a significantly smaller decrease than the control samples ($p<0.01$). Ch and Gl treated samples both displayed a decrease in cell volume ($p<0.01$), from 760±61μm^3 to 451±19μm^3 and 523±27μm^3 respectively, 2h post mechanical trauma when compared to control conditions. Additionally pre-incubation with Ch and Gl resulted in a decrease ($p<0.05$) in relative F-actin (61.93±3.24AU and 35.06±1.30AU respectively) when compared to non-treated samples (77.32±4.72AU) pre-mechanical trauma. Post trauma, samples treated with Ch exhibited a further decrease in relative F-actin followed by a recovery to pre trauma levels (2h post trauma: 39.44±2.84AU; 48h: 65.39±4.82AU). Conversely Gl treated samples exhibited no significant change post trauma ($p>0.05$) and control samples displayed a decrease with no recovery within the 48h post trauma (48h: 48.46±3.76AU). Pro-inflammatory cytokine production was significantly ($p<0.05$) decreased post trauma in samples treated with Ch (IL-1b: Isotonic 121.59±5.90pg/ml/g Ch 106.81±6.82pg/ml/g; MCP-1: Isotonic 1281.57±6.45pg/ml/g Ch 695.75±18.07pg/ml/g; all at 48h post trauma).

These data suggest that despite inducing actin depolymerisation, Ch and Gl exhibit chondro-protective effects by reducing cell volume and decreasing the release of inflammatory cytokines.

Cartilage And Macrophages: Evidence For A Paracrine Loop.

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Osteoarthritis is the most common form of arthritis (Silman, 2008), affecting 10-20% of the UK population over the age of 65 with ~8 million sufferers in the UK (Arthritis Research Campaign; 2008). It is associated with a loss of cartilage integrity due to changes in the regulation of matrix synthesis. Cartilage metabolism is sensitive to cytokines whereby IL-1β and other pro-inflammatory cytokines up-regulate enzymes that breakdown cartilage thus influencing cartilage homeostasis. Cartilage explants have previously been shown to release inflammatory cytokines as a result of single impact trauma, additionally macrophages both release and are induced by changes in inflammatory cytokines. Thus it is of interest to investigate the cytokine feedback loop of macrophages and cartilage as a result of single impact trauma.

Full depth cartilage explants were dissected from the joints of 18-21 month old steers (obtained from a local abattoir with permission). Explants were subjected to a single impact as previously described (Bush *et al.,* 2005) and incubated in 280mOsm/kg H_2O DMEM containing 1% penicillin/streptomycin at 37°C for 4hours. Primary macrophages were isolated from the synovial fluid and incubated for 4 hours with either the supernatant from the impacted explants or with impacted explants and 280 DMEM. Additionally control non-impacted explants and non-treated macrophages were incubated for 4 hours in 280 DMEM. All supernatants were subsequently analysed for inflammatory cytokines by ELISA and expressed as mean±SEM with statistical analysis; (Student T-Test $p<0.05$).

It was found that upon impact of cartilage, there was an up-regulation ($p<0.05$) of both pro-inflammatory (IL-1β: 32.7±3.1 to 53.1±2.2 pg/ml/g, IL-6: 2.5±0.2 to 110.8±2.9 pg/ml/g, IL-8: 35.4±1.8 to 100.7±4.6 pg/ml/g and MCP-1: 40.6±1.9 to 150.3±7.1 pg/ml/g) and anti-inflammatory cytokines (IL-10: 340.4±17.0 to 450.1±29.2 pg/ml/g). Upon stimulation with supernatant from impacted cartilage explants, macrophages were induced to upregulate production of the pro-inflammatory cytokines IL-1β and MCP-1, from 65.5±9.1 to 219.9±23.4 and from 90.2±11.3 to 366.7±27.8 pg/ml/g ($p<0.05$) respectively. In turn this appears to induce a further increase in IL-1β release by cartilage from 53.6±8.9 to 127.3±18.2 pg/ml/g ($p<0.05$).

From these data it is suggested that cytokines released by articular cartilage upon single impact trauma induce an increase in MCP-1 and IL-1β production by macrophages, which in turn induces an additional increase in IL-1β release by articular cartilage, providing evidence for a paracrine cytokine loop between articular cartilage and macrophages.

Bush, P., *et al*., Osteoarthritis and Cartilage, 2005. 13(1): p. 54-65.
Silman AJ, Arthritis and Rheumatism, 2008. 58(5): p. 1220-1225.

Diurnal Inflammatory Cytokine Changes Post-Exercise

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Whilst mechanical impact is essential for cartilage regulation, excessive loading can lead to cartilage damage increasing the risk of Osteoarthritis (OA). Cartilage metabolism is sensitive to cytokines whereby IL-1β and other pro-inflammatory cytokines up-regulate enzyme, including matrix metalloproteases, that breakdown cartilage thus influencing cartilage homeostasis. Given that cartilage matrix composition is known to alter diurnally, specifically in terms of osmolarity this study investigated the effects of exercise on the diurnal expression of pro and anti-inflammatory cytokines.

Venous blood samples were taken at 0h, 2h and 4h post 30min treadmill run at 60% VO_2max from 14 healthy male subjects aged 20-39 years. Subjects underwent anthropometric testing to standardise factors previously shown to effect joint force magnitude, including thigh circumference, body mass and percentage body fat (Browning and Kram, 2007). Exercise was undertaken at 8am (AM) and 6pm (PM) in a cross-over study. A control sample pre-exercise was also taken and inflammatory cytokine concentration (IL-1β, IL-8 & IL-10) determined by Duoset ELISA (R&D Systems) and expressed as mean±SEM. Data was compared using a Student T-Test and deemed significant at $p<0.05$.

Results showed subjects had a body mass of 69.88±3.34kg, a body fat of 11.6±1.2% and a thigh circumference of 50.69±2.24cm. A peak pro-inflammatory response was observed immediately post exercise, and a peak anti-inflammatory response at 2h post exercise for both AM and PM trials. Pro-inflammatory cytokine (IL-1β and IL-8) concentration were seen to increase significantly ($p<0.01$) more after AM exercise than PM (IL-1β: 20.67±2.58% and 9.60±2.35% respectively; IL-8: 25.69±0.53% and 9.01±2.94% respectively), when compared to pre-exercise controls. Conversely, no significant differences were seen in the magnitude of the maximal anti-inflammatory (IL-10) response between AM and PM challenges: 8.97±2.40% and 10.08±2.30% respectively.

These data suggest PM exercise to be more beneficial in terms of reducing exposure to potential risk factors for OA when compared to AM exercise of the same intensity; whereby the reduction in the maximal pro-inflammatory response seen post PM exercise decreases the potential of damaging effects on cartilage metabolism.

Browning RC and Kram R, Medicine and Science in Sports and Exercise, 2007. 39(9): p.1632-1641

Tamoxifen: Chondroprotective Effects Post-Mechanical Trauma.

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Mechanical trauma through repetitive, high-impact sport contributes to the onset of osteoarthritis. Mechanical trauma disrupts cell cytoskeletal organization (Blain, 2009), which is important in maintaining cell integrity. Since tamoxifen has been shown to have anti-calmodulin effects (Hall *et al*., 1999), acting upstream of actin regulatory proteins, this study was designed to investigate whether this compound exerts a chondro-protective effect following mechanical trauma.

Cartilage explants were dissected from the joints of 18-21 month old steers (obtained with permission from a local abattoir) into DMEM. Explants were incubated for 1h in either: isotonic (280mOsm), hypertonic (380 mOsm) or tamoxifen (10μM) supplemented DMEM and subsequently subjected to a single impact as previously described (Bush *et al.,* 2005). Chondrocyte viability, volume and relative F-actin concentration were determined by confocal microscopy and data expressed as mean ± s.e.m; (Student T-test: $p<0.05$), n=45 cells each from 3 distinct experiments.

Explants in isotonic DMEM exhibited a decrease in cell viability from 85±0.05% to 45±2.52% at 48h post impact; $p<0.05$. Conversely, when incubated with hypertonic DMEM or 10μM tamoxifen there was no significant decrease in chondrocyte viability from 90±3.65% and 95±4.51% to 90±2.63% and 88±3.34% respectively at 48h. Tamoxifen incubated samples displayed a decrease in cell volume ($p<0.01$) from 716±23μm^3 to 424±16μm^3 at time 0, when compared to control conditions. However explants incubated in isotonic and hypertonic DMEM all exhibited an impact-induced decrease in cell volume 48hrs post impact, from 716±23μm^3 and 578±31μm^3 to 516±15μm^3 and 302±12μm^3 ($p<0.05$) respectively, whereas tamoxifen incubated explants displayed no significant decrease in cell volume post–impact, from 424±16μm^3 to 432±5μm^3. Additionally pre-incubation with tamoxifen resulted in an decrease ($p<0.05$) in F-actin:volume (0.75±0.04AU) when relative to non-treated samples prior to mechanical trauma, conversely hypertonic pre-incubation displayed an increase ($p<0.05$) in F-actin:volume (1.21±0.06AU). Interestingly, explants pre-treated with isotonic and hypertonic DMEM all exhibited an impact-induced decrease ($p<0.01$) in F-actin:volume to 0.58±0.03AU and 0.73±0.04AU respectively at 48hrs post impact, relative to non-treated samples prior to mechanical trauma. Conversely tamoxifen treated explants showed no significant change in F-actin:volume at 48hrs (0.73±0.04AU).

These data suggest that tamoxifen exhibits a chondro-protective effect in an *in vitro* model of mechanical trauma by inhibiting impact-induced cell volume and F-actin decrease.

REV5901 Induces Calcium Dependant Chondroprotective Effects Post *In Vitro* Mechanical Loading

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Introduction

Mechanical load is essential for cartilage metabolism, providing the necessary signalling for the production of a matrix of sufficient durability to withstand the stress level. However, it has been noted that abnormal loading can lead to cartilage degradation, increasing the risk for the development of osteoarthritis (OA). Abnormal joint loading can lead to an imbalance in cartilage metabolism and activation of IL-1β pathways, promoting synthesis of catabolic proteases and inhibiting ECM synthesis. Mechanical loading has also been observed to result in tissue swelling, indicative of damage to the collagen network, and chondrocyte death by apoptosis and necrosis. However, it was recently observed that cell shrinkage, induced by hyperosmotic challenge, protects against mechanical loading induced chondrocyte death, indicating a role for chondrocyte volume in chondroprotection.

Whilst cartilage is an aneuronal and avascular tissue, chondrocytes are still able to both sense and respond to external stimuli via mechanotransduction. Mechanical loading has been shown to activate mechanotransduction responses by inducing fluid flow, osmotic changes, changes in pressure and alterations to the cell membrane. Volume regulatory mechanisms and Ca2+ signalling pathways have been noted as key components of chondrocyte mechanotransduction whereby Ca2+ assumes the role of an intracellular secondary messenger. It has been observed that mechanical stimuli induces a transient rise in [Ca2+]i, a similar response to that seen during cell volume changes/regulation as a consequence of osmotic challenges.

REV5901 is a 5-lipoxygenase inhibitor, previously demonstrated to be an inhibitor of regulatory volume decrease (RVD) in articular chondrocytes. REV5901 has been observed to have chondroprotective effects post mechanical loading, inducing increased cell viability, decreased cell volume and decreased inflammatory cytokine release. REV5901 has also been shown to induce a sustained rise in [Ca2+]i, suggesting a possible role for Ca2+ signalling in chondroprotective mechanisms post mechanical loading.

This study aims to investigate the role of [Ca2+]i signalling in REV5901 chondroprotective mechanisms post mechanical loading using the [Ca2+]i signalling pathway inhibitors Rottlerin and U73122, and by removal of extracellular Ca2+.

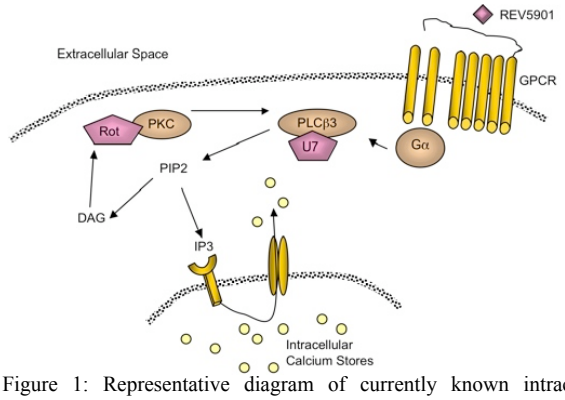

Figure 1: Representative diagram of currently known intracellular calcium signalling pathways (Adapted from Qusous *et al*, *In Press*).

Methods

Cartilage explants were dissected from the metacarpal and metatarsal pharyngeal joints of 18-21 month old bovine steers (obtained from a local abattoir) into 280mOsm/kg H2O DMEM. Explants were incubated for 1h in the presence of 50μM REV5901, supplemented with either 100μM Rottlerin, 100μM U73122 or EGTA. Isotonic (280mOsm), DMSO (1μl/ml) and 50μM REV5901 conditions were used as controls. Post incubation explants were subjected to a single impact as previously described (Bush et al., 2005).

Chondrocytes were visualised by incubation with 5μM Calcein AM and 1μM Propidium Iodide and imaged by confocal laser scanning microscopy. Images were imported into Imaris 7.1 (Bitplane, Netherlands) and subsequently analysed for viability and volume. Supernatant from the explants was analysed for inflammatory cytokines by ELISA at 0h, 2h, 24h and 48h post mechanical impact.

All data expressed as mean ± s.e.m; (Student T-test: *p<0.05), n=45 cells or 6 explants each from 5 distinct experiments.

Results

Pre-treatment with REV5901 induced a chondroprotective effect post mechanical loading, resulting in no significant decrease in cell viability during the 48h following loading, from 93±2.63% at 0h to 86±3.34% at 48h ($p<0.05$). Conversely, both control samples (Isotonic and DMSO conditions) exhibited a decrease in cell viability from 79±0.05% & 83±1.63% at 0h to 45±2.52% & 50±3.45% at 48h; $p<0.05$ and $p<0.05$ respectively. Inhibition of [Ca2+]i signalling with Rottlerin, U73122 and EGTA was observed to block the chondroprotective effects of REV5901, exhibiting a significant decrease in cell viability post mechanical loading from 81±2.12%, 72±2.09% & 88±1.68% at 0h to 59±1.54%, 49±1.98% & 57±2.49% at 48h; $p<0.05$, $p<0.05$ and $p<0.05$ respectively.

Cytokine changes relative to non-loaded controls were significantly increased post mechanical loading in control samples (IL-1β: 1.70±0.18 AU; IL-10: 1.18±0.09 AU), but conversely decreased in samples pre-treated with REV5901 (IL-1β: 0.74±0.21 AU; IL-10: 0.90±0.11 AU). Additional treatment with Rottlerin, U73122 and EGTA also exhibited a decrease in relative cytokine concentration that was not significantly different to REV5901 (IL-1β: 0.75±0.05 AU, 0.72±0.10 AU & 0.70±0.11 AU; IL-10: 0.64±0.12 AU, 0.89±0.08 AU & 0.65±0.09 AU respectively; all at 2h post trauma).

Post mechanical loading, control samples exhibited a decrease in cell volume from 716±38μm3 to 516±39μm3. Samples pre-treated with REV5901 exhibited an initial significant decrease in cell volume when compared to control (716±38μm3 to 552±27μm3; $p<0.01$), but a similar mechanical loading induced decrease (552±27μm3 to 300±35μm3). Samples additionally treated Rottlerin, U73122 and EGTA do not show a significant initial decrease in cell volume when compared to control (716±38μm3 to 670±27μm3, 680±21μm3 & 678±34μm3 respectively), but conversely to control samples do not display a significant load induced decrease in cell volume (670±27μm3 to 656±35μm3, 680±21μm3 to 633±31μm3 & 678±34μm3 to 635±29μm3 respectively).

Discussion

Intracellular calcium is a key regulator of intracellular functions and changes in [Ca2+]i levels have been observed in response to various other mechanical stimuli. REV5901 was shown to induce an elevation in [Ca2+]i, thereby suggesting a potential mechanism by which REV5901 exhibits chondroprotective activity.

Inhibition of [Ca2+]i signalling by Rottlerin, U73122 and EGTA was observed to reduce the chondrotection of REV5901, displaying no maintained cell viability post mechanical loading. However, Rottlerin, U73122 and EGTA did not inhibit the anti-inflammatory effects of REV5901, suggesting this action of REV5901 is not Ca2+ dependant. Conversely Rottlerlin, U73122 and EGTA all prevented cell shrinkage induced by REV5901 and additionally inhibited mechanical load induced cell volume decrease, indicating that REV5901 induced and mechanically induced cell volume changes are Ca2+ dependant.

From these data it can be concluded that REV5901 induces Ca2+ dependant shrinkage of chondrocytes, and Ca2+ independant reduction of inflammatory cytokines, thus resulting in chondroprotection post mechanical loading.